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Phenotypic Consequences of Purine Nucleotide Imbalance in *Saccharomyces cerevisiae*

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ABSTRACT

Coordinating homeostasis of multiple metabolites is a major task for living organisms, and complex interconversion pathways contribute to achieving the proper balance of metabolites. AMP deaminase (AMPD) is such an interconversion enzyme that allows IMP synthesis from AMP. In this article, we show that, under specific conditions, lack of AMPD activity impairs growth. Under these conditions, we found that the intracellular guanylic nucleotide pool was severely affected. *In vivo* studies of two AMPD homologs, Yjl070p and Ybr284p, indicate that these proteins have no detectable AMP, adenosine, or adenine deaminase activity; we show that overexpression of *YJL070c* instead mimics a loss of AMPD function. Expression of the yeast transcriptome was monitored in a AMPD-deficient mutant in a strain overexpressing *YJL070c* and in cells treated with the immunosuppressive drug mycophenolic acid, three conditions that lead to severe depletion of the guanylic nucleotide pool. These three conditions resulted in the up- or downregulation of multiple transcripts, 244 of which are common to at least two conditions and 71 to all three conditions. These transcriptome results, combined with specific mutant analysis, point to threonine metabolism as exquisitely sensitive to the purine nucleotide balance.

THE purine nucleotides, ATP and GTP, are involved in almost all aspects of cellular life. In addition to their role as building blocks of nucleic acids, adenylic and guanylic nucleotides have specific roles. For example, GTP is critical for translation and for signaling through GTPases, while ATP is the major energy-providing molecule in the cell. In yeast, intracellular concentrations of ATP and GTP are clearly different (~5 and 1.5 mM, respectively; Breton *et al.* 2008; Gauthier *et al.* 2008), most probably as the result of regulatory processes that maintain homeostasis. In most eukaryotic cells, including yeast, adenylic and guanylic nucleotides either are synthesized from a common precursor (IMP) or are recycled from preformed bases or nucleosides (Figure 1). While most enzymes involved in these processes have been identified, the physiological consequences of purine nucleotide imbalance are far from being understood. Interestingly, drugs specifically inhibiting GTP synthesis, such as mycophenolic acid (MPA), have a strong immunosuppressive effect and are now widely used to limit allograft rejection. We have previously established the effect of MPA on the yeast proteome (Escobar-Henriques *et al.* 2001) and have identified numerous yeast mutants hypersensitive to this drug (Desmoucelles *et al.* 2002). MPA effects are due to GTP shortage, since they are reversed by exogenous guanine, allowing replenishment of the GTP pool. However, drugs often have secondary effects and can be detoxified and/or diluted during cell growth. As an alternative, consequences of purine nucleotide imbalance can be investigated using yeast mutants. In two previous studies, we have used specific mutants to increase the GTP pool or decrease the ATP pool (Breton *et al.* 2008; Gauthier *et al.* 2008). In this study, we take advantage of a conditional phenotype of an AMP deaminase mutant to revisit GTP shortage consequences in yeast.

AMP deaminase (AMPD; EC 3.5.4.6) is an important enzyme for purine interconversion. During muscle effort, extensive hydrolysis of ATP into ADP results in massive AMP production due to adenylate kinase (myokinase) activity. Under such conditions, AMPD, by draining AMP to IMP, plays a critical role in the stabilization of adenylate energy charge. Consequently, defects in AMPD lead to exercise-induced muscle symptoms such as early fatigue and is the most common muscle enzyme defect in humans (Fishbein *et al.* 1978). While there are three AMPD human isoforms, in *Saccharomyces cerevisiae*, a mutation at a single locus (named *AMD1*; Figure 1) abolishes AMPD activity

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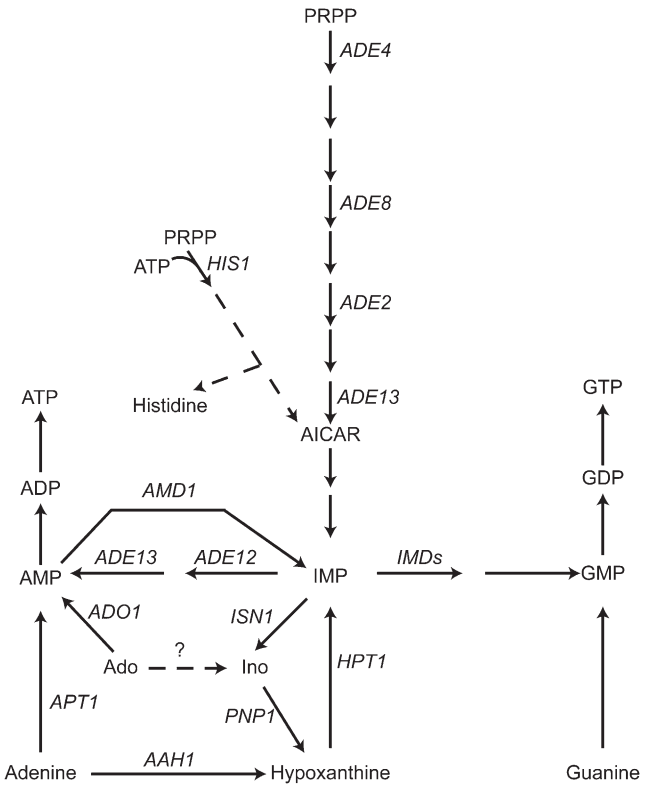

FIGURE 1.—Schematic of purine metabolism in *S. cerevisiae*. Ado, adenosine; AICAR, 5′-phosphoribosyl-5-amino-4-imidazole carboxamide; Ino, inosine; IMP, inosine 5′-monophosphate; and PRPP, 5-phosphoribosyl-1-pyrophosphate. Gene names are italicized. For simplicity, only the enzymatic steps cited in the text are shown in the figure. Putative adenosine deaminase activity is indicated by a question mark.

(MEYER *et al.* 1989). However, two yeast proteins of unknown function (Ybr284p and Yjl070p) are >30% identical to Amd1p in their C terminus (supporting information, Figure S1) (DUENAS *et al.* 1999; VANDENBOL and PORTETELLE 1999). Strikingly, in most organisms, AMPD isoforms have a highly divergent N terminus and in several cases, including yeast, the N terminus of the protein is not required for activity and is often lost during protein purification due to proteolysis (MERKLER *et al.* 1989; SABINA and MAHNKE-ZIZELMAN 2000). Yeast AMP deaminase activity has been characterized *in vitro* (MEYER *et al.* 1989) and is presumed to be high under specific growth conditions where massive synthesis of IMP from AMP has been observed (OSORIO *et al.* 2003; SILLES *et al.* 2005; LORET *et al.* 2007). IMP can then be metabolized through three different pairs of enzymatic reactions. It can give back AMP via two enzymatic steps encoded by the *ADE12* and *ADE13* genes; it can be transformed to GMP via IMP dehydrogenase and GMP synthetase; and, finally, it can be degraded into inosine and hypoxanthine via the successive action of IMP-specific nucleotidase (Isn1p) and purine nucleoside phosphorylase (Pnp1p) (Figure 1) (JONES and FINK 1982; LECOQ *et al.* 2001; ITOH *et al.* 2003). Therefore, yeast AMP deaminase appears as a critical enzyme for both purine interconversion and degradation. While the biochemical properties of yeast AMP deaminase have been well studied (MERKLER *et al.* 1989, 1993; MERKLER and SCHRAMM 1990, 1993), the physiological consequences of a *amd1* defect have not been investigated.

Here, we show that a defect in AMPD activity impairs yeast cell growth under specific conditions, and we establish that AMPD plays a crucial role in maintaining guanylic nucleotide homeostasis. Study of the Amd1p homologs, Yjl070p and Ybr284p, revealed that overexpression of these proteins cannot suppress phenotypes associated with *amd1* deletion. Instead, we show that overexpression of *YJL070c* mimics a loss of Amd1p function. Transcriptome analyses were performed using these various constructs and MPA-treated cells, thus allowing us to evaluate the consequences of guanylic nucleotide depletion obtained through different means. These new genetic tools were also used to revisit the phenotypes of several MPA hypersensitive mutants.

MATERIALS AND METHODS

Yeast media: SD minimal medium contains 0.5% ammonium sulfate, 0.17% yeast nitrogen base Difco, and 2% glucose. SC was prepared as described (SHERMAN *et al.* 1986). SDcasaW is SD medium supplemented with 0.2% casamino acids (Difco) and tryptophan (200 μM). When indicated, adenine, hypoxanthine, and guanine were added at 300 μM and uracil was added at 180 μM.

Yeast strains: All strains belong to, or are derived from, a set of disrupted strains isogenic to BY4741 (*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) or BY4742 (*MAT*α, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) purchased from Euroscarf. The following mutant strains were constructed: Y2077 (*MAT*α, *amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*), Y2362 (*MAT***a**, *ade8::kanMX4, amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*), and Y2693 (*MAT***a**, *his1::kanMX4, ade8::kanMX4, amd1::kanMX4, aah1::kanMX4, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*).

Plasmids: The *IMD2-lacZ* (P354, 2μ *URA3* and P777, 2μ, *LEU2*) and the pCM189 (*CEN*, *URA3*) plasmids used in this study were previously described (GARI *et al.* 1997; ESCOBAR-HENRIQUES and DAIGNAN-FORNIER 2001). The *tet-APT1* (P2091), *tet-YJL070c* (p2483), *tet-HPT1* (P2149), *tet-AMD1* (P2479), and *tet-YBR284w* (P2481) plasmids were obtained by PCR amplification of the corresponding genes using specific oligonucleotides (sequence available upon request) and cloning into pCM189. β-Galactosidase assays were performed as described (KIPPERT 1995).

Transcriptome analysis: For DNA microarray analysis, cells were grown overnight in SDcasaWAU medium, diluted in 50 ml of the same medium, and harvested 24 hr later in exponential phase at A_{600} (absorbance at 600 nm) = 0.6–0.8. RNA were extracted as described in the Gene Expression Omnibus (GEO) entry for this article (GSE9557) and were purified with a RNeasy purification kit (Qiagen) according to the manufacturer's protocol. Normalization was done by the locally weighted scatter plot smoothing (LOWESS) algorithm (BENGTSSON *et al.* 2004). Complete microarray raw data sets and experimental details are available in GEO under accession no. GSE9557. Intracellular nucleotide determination was

performed as previously described (Breton *et al.* 2008; Gauthier *et al.* 2008).

Growth test: Yeast cells were resuspended in sterile water to an $A_{600} = 1$ and submitted to 1/10 serial dilutions. Drops (5 μl) of each dilution were spotted on freshly prepared plates and were incubated at 30° for 48–72 hr.

RESULTS

Deletion of *AMD1* affects growth in the presence of adenine and impairs purine nucleotide balance: While no major growth defect associated with the lack of AMP deaminase activity has been reported previously (Meyer *et al.* 1989), we observed a strong defect in germination and/or growth of *amd1* spores in the presence of adenine (Figure 2A). During vegetative growth, doubling time of the wild-type strain in SDcasa medium (82 min) was not affected by adenine (83 min) while, for the isogenic *amd1* mutant, doubling times were 90 and 140 min in the absence and presence of adenine, respectively. This observation prompted us to measure purine nucleotide pools in these strains. HPLC measurement revealed that adenylic nucleotides were more abundant in the *amd1* mutant, which cannot convert AMP to IMP (Figure 2B). This effect was further enhanced by addition of adenine. Second, we observed that both the GDP and the GTP pools were severely diminished (three- and twofold, respectively) in the *amd1* mutant grown in the presence of adenine compared to the wild-type strain (Figure 2C). In the absence of adenine, GDP and GTP pools were not significantly affected in the *amd1* mutant (Figure 2C). These results establish that Amd1p is critical in maintaining the purine nucleotide balance and suggest that the *amd1* growth defect in the presence of adenine could be due to the inability of this mutant to correctly balance adenylic and guanylic nucleotides.

There are three possible routes to synthesize IMP (and in turn GMP) in the presence of adenine. The first route goes through adenine phosphoribosyltransferase (Apt1p) and AMP deaminase (Amd1p), the second route is through adenine deaminase (Aah1p) and hypoxanthine-guanine phosphoribosyltransferase (Hpt1p), and the third route is *de novo* synthesis from 5-phosphoribosyl-1-pyrophosphate (Figure 1). However, in the presence of adenine, this last route is inhibited due to feedback control on the first enzyme of the pathway (Ade4p) and transcriptional control on all the genes of the *de novo* pathway (Daignan-Fornier and Fink 1992; Rebora *et al.* 2001). Thus, in the presence of adenine and in the absence of Amd1p, IMP and GMP synthesis should mostly take place via Aah1p and Hpt1p and could become limiting for growth. Consistently, the *amd1* growth defect was suppressed by overexpression of *HPT1* (Figure 3A), which drains adenine toward IMP synthesis. On the other hand, the *amd1* growth defect was enhanced by overexpression of *APT1* (Figure 3A), which metabolizes adenine to AMP and therefore competes with Aah1p for available adenine (Figure 1).

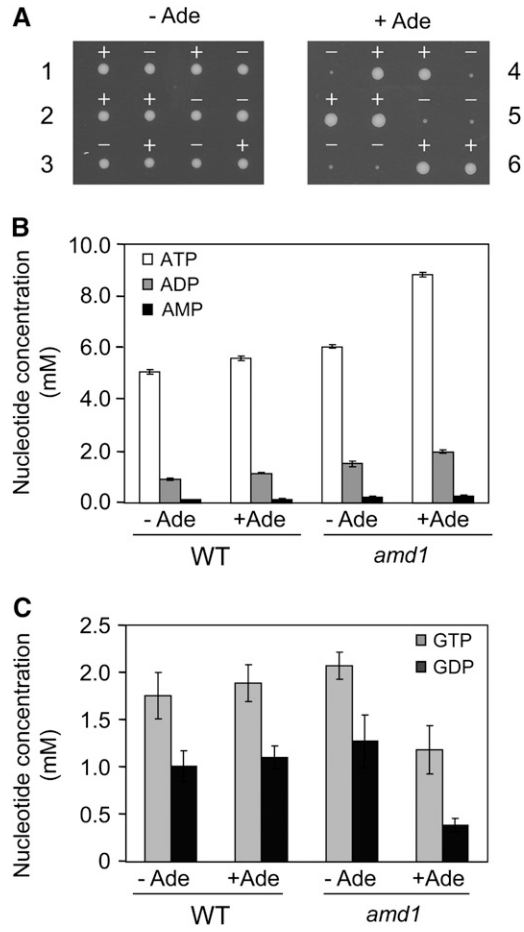

Figure 2.—External adenine affects growth and nucleotide pools of AMP deaminase-deficient strains. (A) Growth of wild-type and *amd1* spores in the absence or the presence of adenine. A heterozygous *AMD1/amd1::KanMX4* diploid strain was sporulated and dissected on SDcasaWU medium containing adenine (+Ade, tetrads 4–6) or not (−Ade, tetrads 1–3). "+" and "−" signs above the colonies refer, respectively, to *AMD1* (+) and *amd1::KanMX4* (−) genotypes determined on the basis of sensitivity or resistance to geneticin associated with *KanMX4* expression. (B and C). Intracellular nucleotide content is affected in the *amd1* mutant grown in the presence of extracellular adenine. Wild-type (WT, BY4742) and *amd1* mutant strains were grown in SDcasaW medium supplemented (+Ade) or not (−Ade) with external adenine. Internal adenylic (B) and guanylic (C) nucleotides were measured as previously described (Breton *et al.* 2008; Gauthier *et al.* 2008).

As expected, an *aah1* mutant, which blocks the second route, also showed a slight growth defect in the presence of adenine (Figure 3A), and consistently, in the case of *aah1*, the growth defect was suppressed by overexpression of *APT1* (Figure 3A), which metabolizes adenine to AMP, the Amd1p substrate (Figure 1).

We then tested the hypothesis that the *amd1* phenotype would be exacerbated by combining it with mutations affecting other IMP-supplying enzymes. Indeed, in the presence of adenine, growth of an *aah1 amd1* double mutant was much more affected than that of each single mutant (Figure 3B). Residual growth in

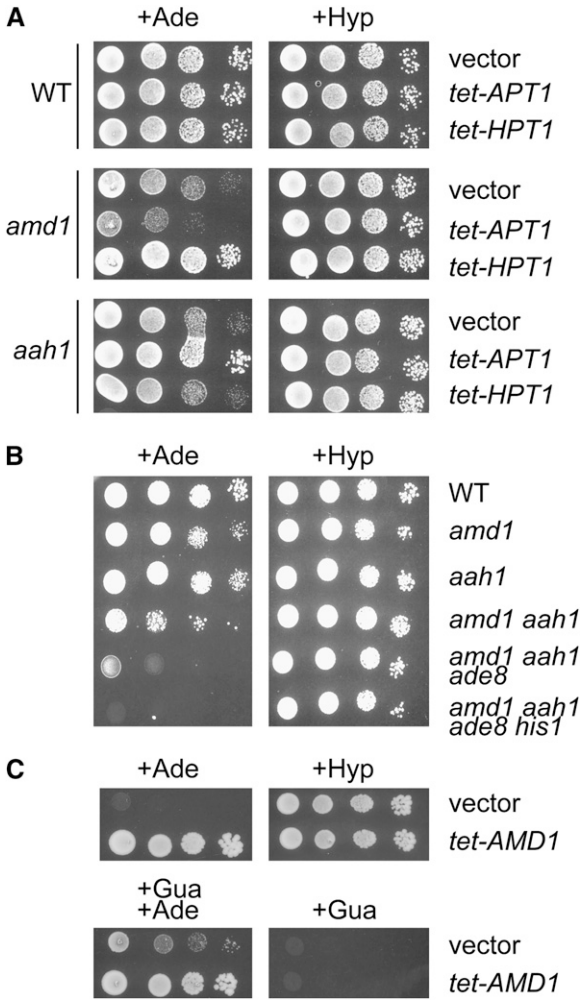

FIGURE 3.—The growth defect of the *amd1* mutant is enhanced by mutations affecting IMP synthesis and partially suppressed by guanine addition. (A) Overexpression of *APT1* exacerbates the growth defect of the *amd1* mutant in the presence of external adenine. Cells were transformed with the pCM189 plasmid (vector), *tet-APT1*, or *tet-HPT1*. Transformants were serial diluted and spotted on SDcasaW medium supplemented with adenine (+Ade) or hypoxanthine (+Hyp). (B) Combinations of the *amd1* mutation with *aah1*, *ade8*, and *his1* lead to drastic decrease of growth in the presence of adenine. (C) Growth defect of the *amd1 aah1 ade8 his1* mutant in the presence of adenine is due to guanylic nucleotide starvation. The quadruple *amd1 aah1 ade8 his1* mutant was transformed with the pCM189 empty plasmid (vector) or the plasmid allowing overexpression of *AMD1* (p2479). Cells were spotted on SDcasaW medium supplemented with adenine (+Ade), guanine (+Gua), or hypoxanthine (+Hyp), as indicated.

this mutant was due to IMP synthesis via AICAR from the purine and histidine *de novo* pathways (REBORA *et al.* 2005) (Figure 1) and could be totally abolished in the *ade8 his1 aah1 amd1* quadruple mutant (Figure 3B). As expected, this quadruple mutant was fully viable when hypoxanthine was provided as a purine source (Figure 3B). Furthermore, growth of the quadruple mutant in the presence of adenine was restored to a certain extent by the addition of guanine, indicating that the growth defect is, at least in part, due to guanylic nucleotide shortage (Figure 3C). Incomplete suppression by guanine could be due to the fact that guanylic nucleotide shortage is not the sole problem faced by this strain. Alternatively, it could be due to poor guanine uptake in the presence of adenine since both compounds are transported by the purine–cytosine permease (SCHMIDT *et al.* 1984).

Overexpression of *YJL070c* phenocopies a *amd1* knock-out mutant: As mentioned above, the function of the two *AMD1* homologs, *YBR284w* and *YJL070c*, is unknown. Importantly, both proteins lack several residues (Figure S1) conserved in all described purine deaminases (RIBARD *et al.* 2003). Overexpression of these genes could not rescue growth of the quadruple *aah1 ade8 amd1 his1* mutant in the presence of adenine (Figure S2A), indicating that these genes do not significantly contribute to AMPD activity. It is notable that these genes do not contribute to adenosine or adenine deaminase activity (Figure S2, A and B). Finally, mutations in these two genes, either alone or combined with *amd1*, did not result in additional phenotypes (Figure S2C). However, to our surprise, we found that overexpression of *YJL070c*, but not that of *YBR284w*, affected the growth of a wild-type strain, specifically in the presence of adenine (Figure 4A). This phenotype is reminiscent of the one found for the *amd1* mutant (Figure 2A). Importantly, the effect of *YJL070c* was dependent on the presence of Amd1p, since *YJL070c* overexpression had no additional effect in the *amd1* deletion mutant (Figure 4A). We thus investigated whether overexpression of *YJL070c* would mimic other phenotypes of the *amd1* knock-out. Indeed, the phenotype, associated with *YJL070c* overexpression, was strongly enhanced in the *aah1* mutant (Figure 4A), as found for the *amd1* mutation (Figure 3B). Measurement of guanylic nucleotide pools revealed that overexpression of *YJL070c* resulted in a strong decrease of both GDP and GTP intracellular concentration (Figure 4B). This effect was observed only when cells were grown in the presence of adenine as for the *amd1* mutant (Figure 2C). In yeast, a guanylic nucleotide limitation results in strong induction of *IMD2* gene expression (ESCOBAR-HENRIQUES and DAIGNAN-FORNIER 2001). Clearly, overexpression of *YJL070c* as well as the *amd1* deletion resulted in strong induction of *IMD2-lacZ* expression only in the presence of adenine (Figure 4, C and D), while no effect was observed in the *ybr284w* and *yjl070c* mutants (Figure 4D). Therefore, according to all tested criteria, overexpression of *YJL070c* phenocopies a *amd1* deletion.

Transcriptome analysis of guanylic nucleotide-depleted yeast cells: The *amd1* mutation and the overexpression of *YJL070c* provide us with new ways to challenge cells with guanylic nucleotide depletion. We took advantage of these strains to evaluate the effects of GDP and GTP limitation on the yeast transcriptome and to compare

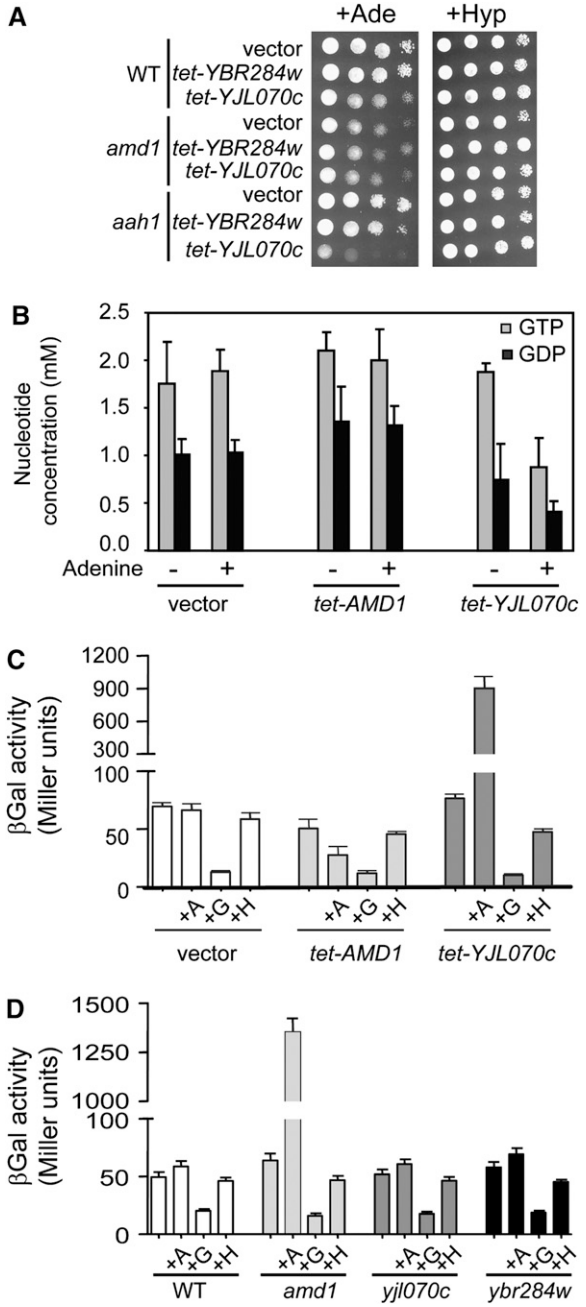

FIGURE 4.—Overexpression of *YJL070c* phenocopies the *amd1* deletion mutant. (A) Effect of *YJL070c* or *YBR284w* overexpression on growth in the presence of adenine or hypoxanthine. Cells were transformed with the pCM189 empty plasmid (vector) or either the *tet-YBR284w* or *tet-YJL070c* plasmids. (B) Intracellular guanylic nucleotide content is specifically decreased in wild-type cells overexpressing *YJL070c* and grown in the presence of adenine. Wild-type (BY4742) cells were transformed with the pCM189 vector or plasmids allowing overexpression of *AMD1*, *YJL070c*, or *YBR284w*. Transformants were grown in SDcasaW medium supplemented or not with external adenine, and intracellular guanylic nucleotide content was measured as previously described (BRETON *et al.* 2008). (C) Overexpression of *YJL070c* leads to derepression of the *IMD2-LacZ* fusion expression. Cells were cotransformed with *IMD2-lacZ* plasmid and either the vector or the plasmids allowing overexpression of *AMD1* or *YJL070c*. Transformants were grown in SC medium supplemented or not with adenine (+A), guanine (+G), or hypoxanthine (+H). (D) Expression of *IMD2-LacZ* fusion is drastically increased in a *amd1* mutant in the presence of adenine. Cells were transformed with a plasmid carrying a *IMD2-lacZ* fusion. Transformants were grown in SDcasaW medium supplemented or not with adenine (+A), guanine (+G), or hypoxanthine (+H).

them to those of the immunosuppressive drug MPA, which specifically inhibits eukaryotic IMP dehydrogenase (IMPDH). In MPA-treated cells, GDP and GTP concentrations were severely affected in a dose-dependent manner, while adenylic nucleotide pools were only slightly affected (Figure 5A). Importantly, MPA treatment also led to massive accumulation of IMP, the substrate of IMPDH, and its nucleoside (inosine) and base (hypoxanthine) derivatives (Figure 5A). It is notable that the ~50% decrease in GTP concentration found in both the *amd1* mutant and the *YJL070c* overexpressing strains (Figure 2C and Figure 4B) is intermediary to the 17% and 80% found for the lower and higher MPA concentrations, respectively.

The effects of guanylic nucleotide shortage on the yeast transcriptome were then evaluated in a wild-type strain treated (or not) with MPA (0.03 and 0.1 mg/liter) in a *amd1* mutant and in a wild-type strain overexpressing (or not) *YJL070c*. The first conclusion was that MPA treatment affected expression of multiple genes that were either up- or downregulated. The number of affected genes was much higher at 0.1 mg/liter MPA compared to 0.03 mg/liter (Figure 5B). While most of the 51 genes affected at low MPA concentration are also affected at the higher concentration, 14 (27%) were not, suggesting that the transcriptional response to the drug is not simple.

Comparison of the transcriptome in *YJL070c* overexpression and *amd1* deletion strains to their cognate control strains revealed an altered expression for 358 and 407 genes, respectively, 246 of which were affected in both conditions (Figure 5C, Table S1). This result strongly supports our genetics and biochemical data, indicating that overexpression of *YJL070c* phenocopies the *amd1* deletion. When looking at the five most affected genes in each condition (Figure 5D), several conclusions can be drawn. First, in all four conditions, *IMD2* expression was strongly upregulated (>10-fold), thus confirming the *IMD2-lacZ* results (Figure 5D). Second, some genes, such as *SSA4* or *HSP82*, were strongly and specifically affected by MPA, even at the lower dose, but did not respond to GTP shortage induced by *amd1* or *tet-YJL070c*. Because these genes are involved in the stress response, this could reflect a side effect of MPA not directly linked to GTP synthesis inhibition. Reciprocally, genes such as *ICY2* or *LEU4* strongly responded to the *amd1* deletion and *YJL070c* overexpression but not to MPA treatment. Finally, in most cases (*LYS1*, *ARG1*, *CPA2*, *SNZ1*, *TMT1*, *HIS4*), the

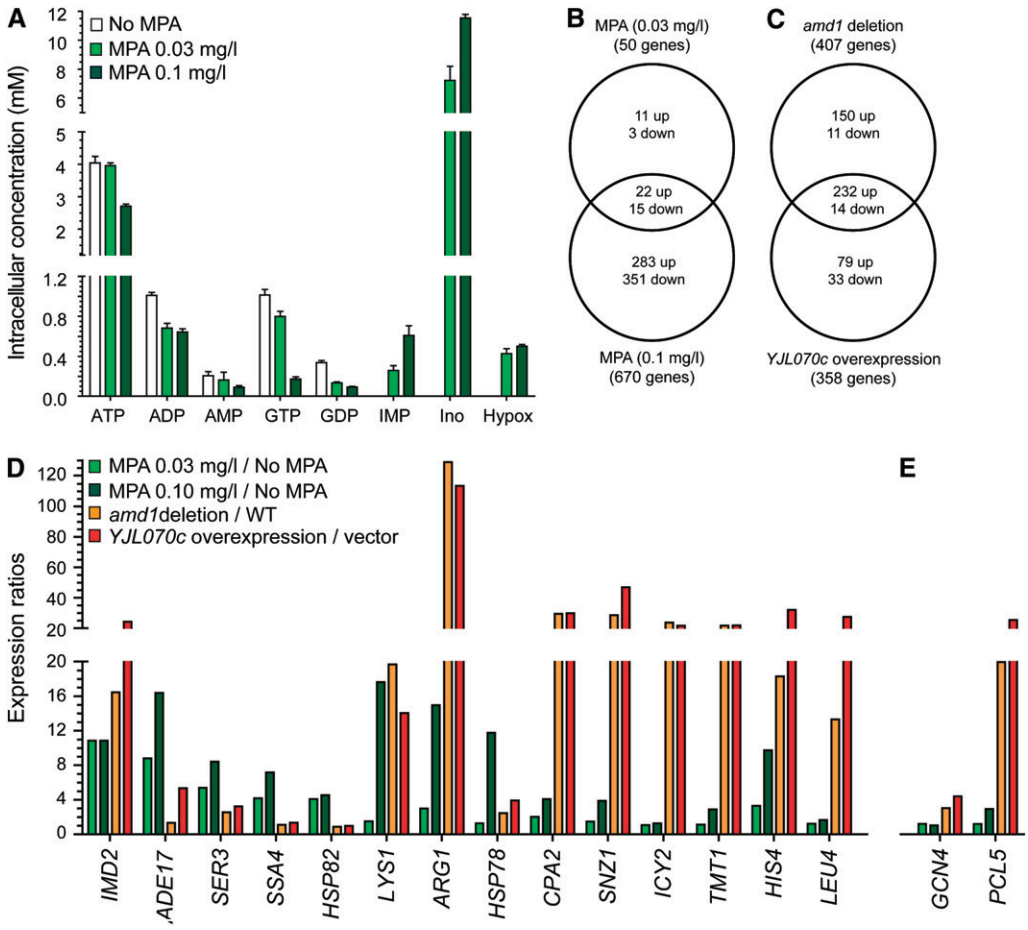

FIGURE 5.—Global transcriptional response to *amd1* deletion, *YJL070c* overexpression and MPA treatment in the presence of adenine. (A) Intracellular nucleotide content is affected by MPA treatment. Wild-type cells were grown in SDcasaWUA medium supplemented or not with MPA. Internal adenylic and guanylic nucleotides were measured as previously described (BRETON *et al.* 2008; GAUTHIER *et al.* 2008). (B and C) Transcriptional response to guanylic nucleotide depletion obtained by MPA treatment, *amd1* deletion, and *YJL070c* overexpression. Transcriptional response was monitored by microarray analyses. Venn diagrams show numbers of genes differentially expressed (by a factor ≥2 compared to untreated wild type) in response to various MPA concentrations (B) in a *amd1* mutant and a strain overexpressing *YJL070c* (C). (D) Expression ratios of the five most upregulated genes for each comparison set used in the transcriptome analysis. (E) Expression of *GCN4* and *PCL5* in the various conditions.

transcriptional response seems gradual, low at the low MPA dose, higher at 0.1 mg/liter MPA, and even higher when *amd1* is mutated or *YJL070c* overexpressed. This could appear surprising since the high dose of MPA treatment leads to lower GTP concentrations, but could reflect secondary effects of the drug. Interestingly, many of these genes are under the control of the transcriptional factor Gcn4p, and it should be noted that *GCN4* itself is transcriptionally induced in the *amd1* mutant and under the *tet-YJL070c* conditions but is unaffected in the MPA-treated cells (Figure 5E). Furthermore, *PCL5*, a major regulator of Gcn4p stability (SHEMER *et al.* 2002), is also differentially induced (Figure 5E) and could negatively modulate *GCN4* overexpression. Global comparison revealed 71 genes similarly affected by *amd1* knock-out, *YJL070c* overexpression, and 0.1 mg/liter MPA treatment (Figure S3), most of which are involved in amino acid metabolism and regulated by Gcn4p (NATARAJAN *et al.* 2001). Thus, although *GCN4* itself responds differently under the various conditions, GTP shortage is associated with upregulation of multiple *GCN4* target genes.

***Tet-YJL070c* as a tool to revisit MPA hypersensitive mutants:** Our earlier search for yeast mutants sensitive to MPA treatment had revealed eight amino acid metabolism mutants (DESMOUCELLES *et al.* 2002). Since our transcriptome analysis showed strong induction of several such amino acid metabolism genes when guanylic nucleotides are scarce, we used *YJL070c* to reinvestigate this phenotype. The *tet-YJL070c* construct was found to severely affect the growth of three mutants (*tat1*, *thr1*, and *hom2*) on adenine; it had a weaker effect on the *ilv1*, *bap1*, *ser2*, and *hom6* mutants, and no major effect on *ser1* (Figure 6A). As a control experiment, we showed that a *amd1 thr1* double mutant behaved just as the *thr1* mutant carrying the *tet-YJL070c* plasmid did (Figure S4). Importantly, the *hom2* and *thr1* growth defects, associated with *YJL070c* overexpression, were suppressed by the addition of threonine in large amounts to the growth medium (Figure 6B). Since both *hom2* and *thr1* mutations block threonine biosynthesis, this result strongly suggests that threonine uptake is limiting for growth in these mutants when guanylic nucleotides are scarce. Thus, this phenotypic analysis supports the idea that

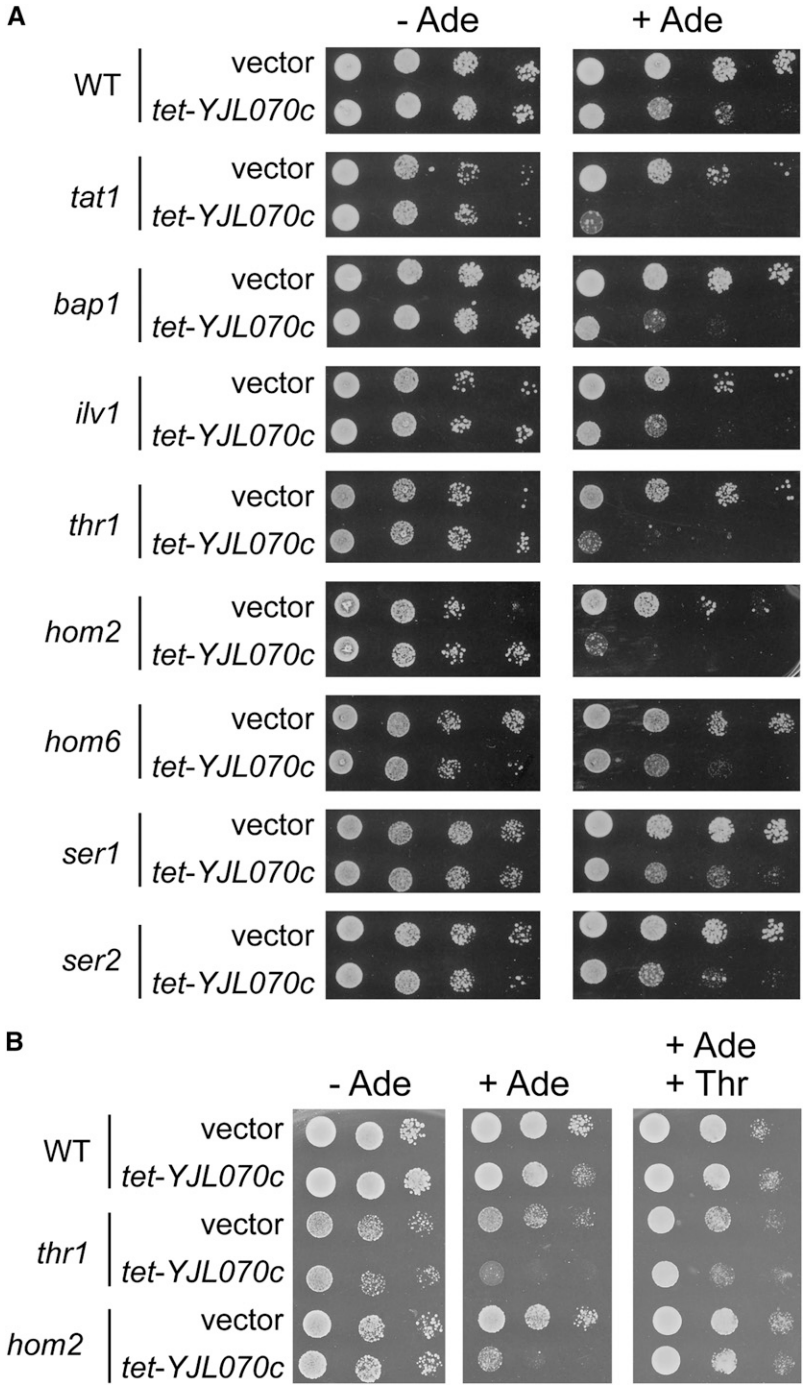

FIGURE 6.—*YJL070c* overexpression severely affects growth of threonine biosynthesis mutants in the presence of adenine. Cells transformed with pCM189 (vector) or *tet-YJL070c* were spotted on SDcasaW medium supplemented (+ Ade) or not (− Ade) with adenine and threonine (+ Thr) as indicated.

there is a connection between guanylic nucleotide limitation and amino acid metabolism.

In yeast, hypersensitivity to MPA is often used as a criterion to identify transcriptional elongation mutants, the rationale being that GTP shortage (substrate limitation) combined to an elongation default affects transcription efficiency and/or accuracy so severely that it results in growth impairment. However, in several of these MPA-sensitive mutants, such as *dst1* (*ppr2*), expression of *IMD2*, which encodes the MPA-resistant isoform of IMPDH (HYLE *et al.* 2003), is drastically decreased (SHAW and REINES 2000). Thus, there are two major and nonexclusive hypotheses to explain why these mutants are hypersensitive to MPA. The first one is that sensitivity to MPA could be due to *IMD2* low expression. The second one is that the mutants could be sensitive due to the combination of low GTP concentration and altered transcription elongation. The *tet-YJL070c* construct, because it allows decreasing GTP concentration independently of MPA, was used to attempt to settle this question.

Consistent with the first hypothesis, expression of *IMD2* under a heterologous *tet* promoter reversed MPA hypersensitivity of the *dst1* mutant strain (Figure 7A),

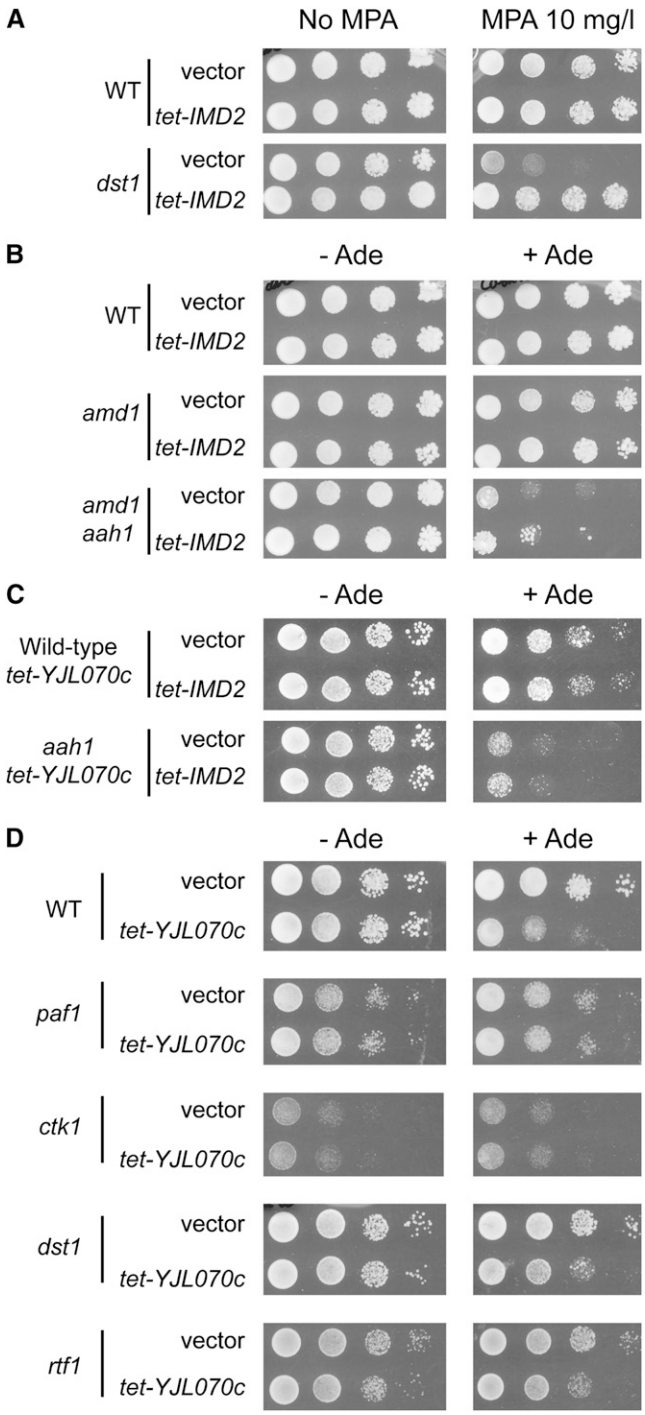

Figure 7.—Effect of guanylic nucleotide limitation on transcription mutants. Cells were transformed with pCM189 (vector) or the *tet-IMD2* plasmid, and transformants were spotted on SDcasaW medium supplemented or not with MPA (10 mg/liter) (A) or with adenine (Ade) as indicated (B). (C) Cells containing the *tet-YJL070c* overexpression plasmid were transformed with pCM189 (vector) or the *tet-IMD2* plasmid, and transformants were spotted either on SC medium lacking uracil and leucine (C) or on SDcasaW (D).

and, importantly, it did not significantly suppress the adenine-specific growth defect of the *amd1* mutant, although it slightly improved growth of all the strains in the presence of adenine (Figure 7B). Thus, although both MPA treatment and *amd1* mutation induce *IMD2* expression (Figure 4 and Figure S3B), the growth defect associated with the *amd1* mutation is independent of *IMD2* expression. Similarly, *IMD2* overexpression had no effect on the growth defect due to overexpression of *YJL070c* in the presence of adenine (Figure 7C).

To test the second hypothesis and establish whether a decrease of GTP concentration in various transcription mutants was sufficient to explain their hypersensitivity to MPA, we took advantage of the *tet-YJL070c* plasmid to decrease GTP pools independently of MPA treatment. We thus overexpressed *YJL070c* in various MPA hypersensitive mutants proposed to affect transcription elongation and monitored growth and GTP concentration. Strikingly, while overexpression of *YJL070c* in the mutants and in the isogenic wild-type strain similarly affected GTP levels (Figure S5), it had no drastic effect on growth in the presence of adenine (Figure 7D). It is important to note that the double *amd1 dst1* mutant behaved just as the *dst1* mutant carrying the *tet-YJL070c* plasmid did (Figure S4). Together, our results indicate that a significant part of the MPA sensitivity of these mutants is most likely due to poor expression of *IMD2* rather than to a synthetic growth defect due to impaired transcription elongation combined with GTP limitation. However, it cannot be excluded that the growth defect of these transcription mutants observed in the presence of MPA could necessitate a more severe GTP limitation than the one caused by *YJL070c* overexpression in the presence of adenine.

DISCUSSION

A major conclusion from our results is that, in yeast, a defect in AMP deaminase is associated with a severe GDP/GTP pool depletion. This effect could be observed only in the presence of adenine and under conditions where the *de novo* pathway is turned down. We interpret this result as follows: in the absence of adenine, IMP synthesized from the *de novo* pathway is sufficient to provide wild-type levels of GDP and GTP. Addition of adenine results in a strong downregulation of the *de novo* pathway (Daignan-Fornier and Fink 1992) and a subsequent decrease of IMP synthesis, which, in the *amd1* mutant, cannot be fully compensated for by IMP synthesis via adenine deaminase. Interestingly, a recent report on *Arabidopsis thaliana* AMP deaminase inhibition by deaminoformycin showed a synergistic effect of adenine on deaminoformycin toxicity (Xu *et al.* 2005). This phenotype is highly similar to the inhibitory effect of adenine on growth of the *amd1* mutant. However, in *A. thaliana*, adenylic nucleotide accumulation appears to be the initial cause of growth inhibition; by contrast, in yeast, partial reversion of the adenine effect by guanine (Figure 4B) suggests that guanylic nucleotide shortage is, at least in part, responsible for growth inhibition. In our previous work, we

have studied the transcriptional response to ATP limitation (*adk1* mutant; Gauthier *et al.* 2008) and GTP overproduction (constitutive *HPT1* mutant; Breton *et al.* 2008). In this work, we now document the transcriptional response to GTP shortage obtained by different means. Importantly, while all these conditions result in purine nucleotide imbalance, there is no evidence for a common transcriptional response. Furthermore, GTP shortage and overproduction do not result in opposite transcriptional responses.

In this study, important data were collected on the yeast AMP deaminase gene family. On the basis of phenotypic analysis, we established that none of the two *AMD1* homologs, *YBR284w* and *YJL070c*, encodes AMP, adenosine, or adenine deaminase activity (Figure S2). These results are in agreement with the fact that both proteins lack important residues (Figure S1) conserved in all described purine deaminases (Ribard *et al.* 2003). However, because these genes are syntenic with genes in other fungi (*S. paradoxus*, *S. bayanus*, *Ashbya gossypii*, etc.), and because the Yjl070p protein has been detected by mass spectrometry (Ghaemmaghami *et al.* 2003), it seems unlikely that these genes could be pseudogenes. Concerning Yjl070p, we favor the hypothesis that it could have a noncatalytic regulatory function. Indeed, we found that overexpression of Yjl070p, in the presence of adenine, has a strong effect on guanylic nucleotide concentration, and growth was impaired. These results are highly similar to those obtained with the *amd1* deletion, indicating that overexpression of *YJL070c* mimics the *amd1* knock-out. Importantly, Amd1p and Yjl070p copurified in a global proteome interaction analysis (Krogan *et al.* 2006), thus suggesting a possible direct interaction that could affect AMP deaminase activity. An attractive working hypothesis would be that Yjl070p could form inactive heterodimers with Amd1p; however, our attempts to document a direct inhibitory effect *in vitro* were unsuccessful (C. Saint-Marc and B. Daignan-Fornier, unpublished data). The fact that *YJL070c* overexpression mimicked the *amd1* deletion was confirmed by our transcriptome analysis showing that a large set of common genes were induced in both conditions. The transcriptional response to MPA was more divergent. This could be due to the fact that MPA treatment, by inhibiting IMPDH, leads to massive accumulation of inosine ($\leq$12 mM) as well as IMP and hypoxanthine, although to a lesser extent. No accumulation of these compounds was observed in the *amd1* mutant or *YJL070c* overexpression strains.

We also took advantage of GTP limitation induced by *YJL070c* overexpression to revisit the proposed phenotypical link between GTP shortage and transcription elongation mutants, which are hypersensitive to MPA. We found that all tested mutants (*paf1*, *dst1*, *rtf1*, and *ctk1*) were not more affected than the wild-type control by *YJL070c* overexpression. Interestingly, a study of transcription elongation *in vivo* on a specific gene indicated that *dst1*, *rtf1*, and *ctk1* mutations, on their own, do not affect transcription elongation or processivity (Mason and Struhl 2005). We therefore favor the hypothesis that MPA hypersensitivity of these mutants is due to the need for these factors in MPA-induced *IMD2* upregulation and is not a secondary effect of low GTP concentrations upon transcription *per se*. These results cast a doubt upon the use of MPA sensitivity as a criterion in identifying transcription elongation mutants.

Our transcriptome data revealed 71 genes affected at least twofold in all three conditions leading to GDP/GTP shortage (Figure 6B). Strikingly, many of these genes are involved in amino acid metabolism, and the vast majority of them are under the control of the Gcn4p transcription factor. This result is in good agreement with previous reports showing induction of *GCN4* target genes in response to severe purine limitation (Rolfes and Hinnebusch 1993). An important result is that some amino acid metabolism mutants appear exquisitely sensitive to guanylic nucleotide limitation. We found that the threonine biosynthesis mutants, *thr1* or *hom2*, are strongly affected by *YJL070c* overexpression and that this growth defect could be alleviated by increasing external threonine concentration. Interestingly, *thr1* and *hom2* mutants are hypersensitive to hydroxyurea (Hartman and Tippery 2004), and threonine metabolism has been shown to be important for dNTP pool homeostasis in yeast (Hartman 2007); however, the precise mechanism leading to this cross-pathway effect is not known. In addition, both *thr1* and *hom2* mutants showed reduced fitness under various growth conditions (Giaever *et al.* 2002), suggesting that threonine availability could be central in responding to different cellular perturbations. Together, our results point to amino acid uptake as limiting under guanylic nucleotide shortage, thus revealing a new metabolic crosstalk that could be relevant for understanding the immunosuppressive and antiproliferative effects of mycophenolate derivatives.

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GENETICS

Supporting Information
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Phenotypic Consequences of Purine Nucleotide Imbalance in *Saccharomyces cerevisiae*

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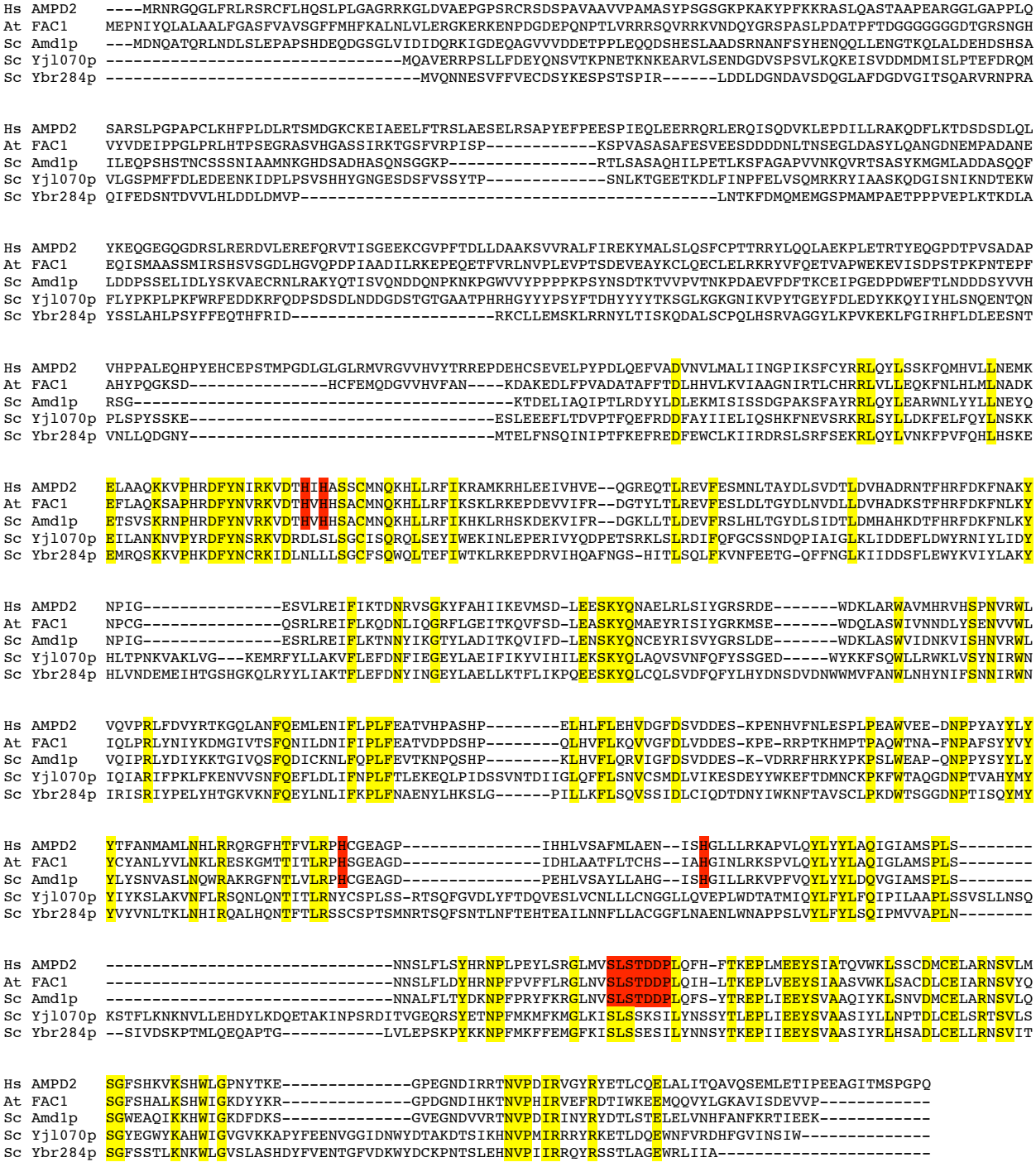


FIGURE S1.—Sequence comparison of AMP deaminase homologs. Sequences of human AMP deaminase isoform 2 (AMPD2, NP_004028), */Arabidopsis thaliana/* FAC1 (NP_850294) and yeast Amd1p (NP_013677), Yjl070p (NP_012465) and Ybr284p (NP_009843) were aligned using ClustalW. Residues conserved in all five proteins are highlighted in yellow. Dashes indicate the gaps created for alignment. The four histidine residues involved in zinc binding and the SL(S/N)TDDP motif highly conserved in adenosine deaminases are highlighted in red.

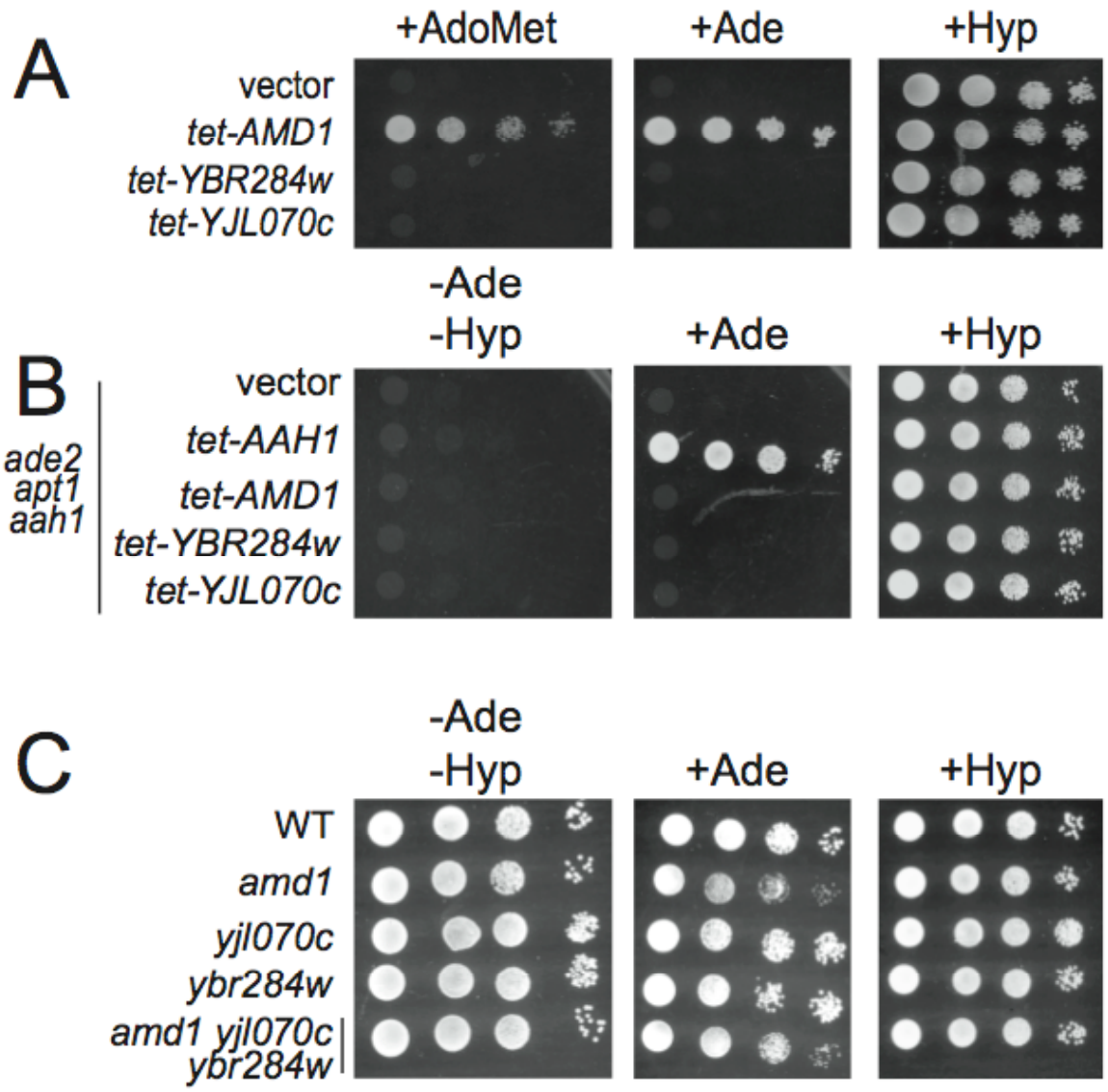
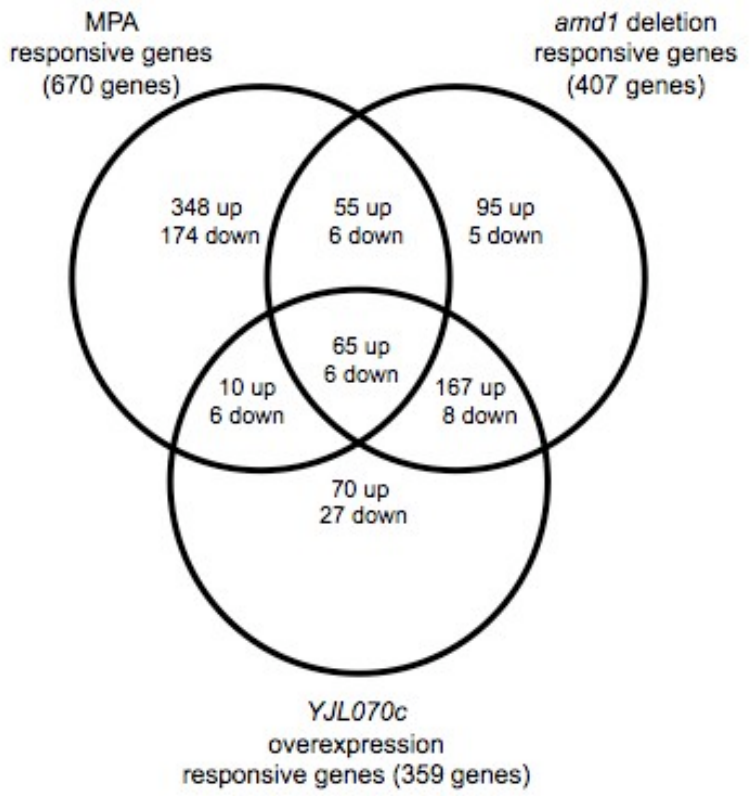


FIGURE S2.—Overexpression of *YBR284w* and *YJL070c* cannot bypass the lack of AMP-, adenosine-, or adenine-deaminase activities *in vivo*. (A) Overexpression of *YJL070c* or *YBR284w* does not allow utilization of S-adenosyl- methionine (AdoMet) or adenine as a purine source in the quadruple *amd1 aah1 ade8 his1*. To be utilized as a purine source, AdoMet requires adenosine deaminase (question mark in Figure 1) or adenosine kinase (ADO1 figure 1) + AMP deaminase activities. Clearly, overexpression of *YJL070c* or *YBR284w* does not provide activities required for AdoMet utilization, while *AMD1* does. (B) Overexpression of *YJL070c* or *YBR284w* does not allow utilization of adenine by an *aah1* mutant lacking adenine deaminase activity. (C) Growth of wild-type, *amd1*, *yjl070c* and *ybr284w* mutant strains in the presence of external purine. Serial dilutions of yeast cells in exponential growth-phase were spotted on SD casaW medium containing uracil and supplemented or not with adenine (+Ade) or hypoxanthine (+Hyp).

A



B

AAD10; ADE3; ARG1;ARG3; ARG4; ARG5,6; ARG8; ARO9; ***ATF2****; BNA1; BOP2; BSC5; CLG1; CPA1; CPA2; DDR48; DLD3; ECM13; ECM17; ECM40; GCV2; GGC1; HIS4; HIS5; HIS7;* ***HO****; HOM3; HSP78; IDH2; ILV2; IMD1; IMD2;* ***INO1****; KRS1; LYS1; LYS20; MET1; MET10; MET13; MET16; MET17; MET2; MET28;* ***MF(ALPHA)2****; NCE103; PCL5;* ***PTR2****; PYC1; PYC2; Q0017; RIB3;* ***SAM2****; SER3; SNO1; SNX41; SNZ1; STE2; THI20; THI7; TMT1; URA10; VHT1; YAR075W; YAT2; YDL025C; YGL059W; YGL117W; YGR110W; YJL213W; YMR173W-A; YOR302W*

FIGURE S3.—(A) Distribution of differentially expressed genes (by a factor 2 or more) in wild-type cells compared to wild-type cells treated with MPA 0.1 mg/l for 60 min(left), to *amd1* deleted mutant (right) or to *YJL070c* overexpression (bottom). (B) List of genes for which expression is up- or down-regulated in all three conditions cited in (A). Gene names in bold correspond to down-regulated genes and gray boxed genes correspond to genes regulated by the Gcn4p transcription factor.

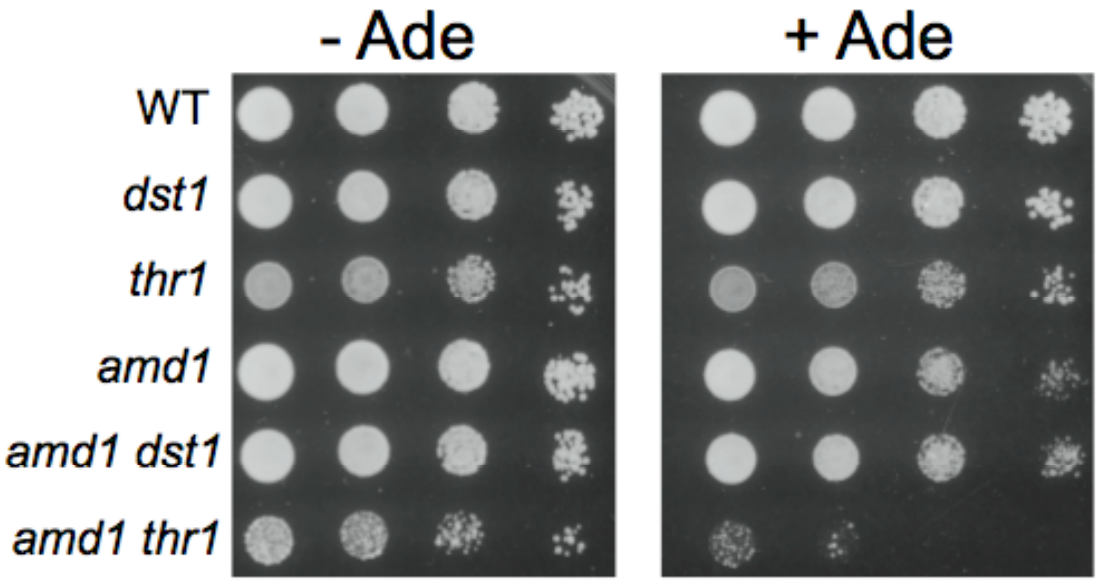


FIGURE S4.—Growth of various mutant strains in the presence or absence of adenine.

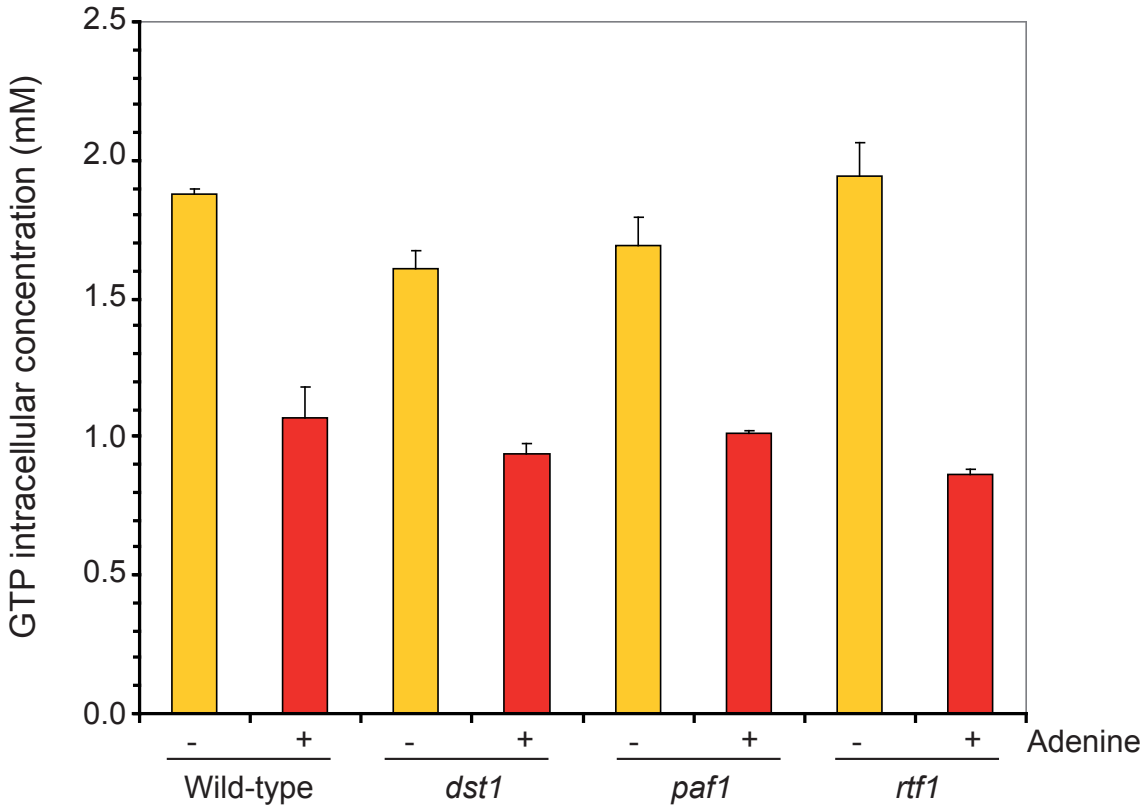


FIGURE S5.—GTP Intracellular concentration in wild-type and mutant strains. Cells were transformed with the plasmid allowing *YJL070c* overexpression. Transformants were grown in SDcasaW medium supplemented (+) or not (-) with external adenine and intracellular GTP content was measured as previously described (BRETON et al. 2008).

TABLE S1

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.109.105858/DC1.

All results presented in this file correspond to genes up-regulated (Ratio> 2) or down-regulated (Ratio <0,5) by at least a factor 2. For all experiments, the entire data sheets are available at
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xvkthqaquiemkji&acc=GSE9557

Legend of column titles presented in tables of the different data sheets is:

Ratio R = Cy5/Cy3
Mnorm is the log base 2 of the ratio : M = log2(Cy5/Cy3)
SD Mnorm is the variation
A is the average of the log base 2 of the intensities : A = (log2(Cy3 intensity) + log2(Cy5 intensity)) / 2