The Isolation and Characterization of *Saccharomyces cerevisiae* Mutants That Constitutively Express Purine Biosynthetic Genes

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ABSTRACT

In response to an external source of adenine, yeast cells repress the expression of purine biosynthesis pathway genes. To identify necessary components of this signalling mechanism, we have isolated mutants that are constitutively active for expression. These mutants were named *bra* (for bypass of repression by adenine). *BRA7* is allelic to *FCY2*, the gene encoding the purine cytosine permease and *BRA9* is *ADE12*, the gene encoding adenylosuccinate synthetase. *BRA6* and *BRA1* are new genes encoding, respectively, hypoxanthine guanine phosphoribosyl transferase and adenylosuccinate lyase. These results indicate that uptake and salvage of adenine are important steps in regulating expression of purine biosynthetic genes. We have also shown that two other salvage enzymes, adenine phosphoribosyl transferase and adenine deaminase, are involved in activating the pathway. Finally, using mutant strains affected in AMP kinase or ribonucleotide reductase activities, we have shown that AMP needs to be phosphorylated to ADP to exert its regulatory role while reduction of ADP into dADP by ribonucleotide reductase is not required for adenine repression. Together these data suggest that ADP or a derivative of ADP is the effector molecule in the signal transduction pathway.

MICROORGANISMS alter their metabolism in response to the presence of metabolic precursors in the environment. This adaptation requires the ability to sense nutrient levels and then transduce a signal to redirect the synthesis of metabolic enzymes. We are interested in the signalling cascade that leads to repression by adenine in *Saccharomyces cerevisiae*.

Coordinate repression of *de novo* purine synthesis genes has been reported in bacteria and bakers yeast (MOMOSE *et al.* 1966; NEUHARD and NYGAARD 1987; DAIGNAN-FORNIER and FINK 1992). Interestingly, this repression is achieved by very different processes in *Escherichia coli* and *Bacillus subtilis*. In both bacteria the regulation is mediated by a specific repressor named purR. Although in *E. coli* binding of the repressor to its 16-bp target site depends on the presence of hypoxanthine or guanine (ROLFES and ZALKIN 1990), in *B. subtilis* the repressor binding site is 110 bp long and its interaction with the regulatory protein is inhibited by 5-phosphoribosyl 1-pyrophosphate (PRPP) (WENG *et al.* 1995).

In yeast adenine repression is less well understood. We have previously shown (DAIGNAN-FORNIER and FINK 1992) that expression of several genes of the purine biosynthetic pathway is repressed in the presence of adenine in the growth medium. Derepression requires the transcription factors Bas1p and Bas2p. Both factors bind to the promoters of purine biosynthetic (*ADE*) genes. Although Bas2p is involved in multiple metabolic pathways (BRAUS *et al.* 1989; VOGEL *et al.* 1989; BRAZAS and STILLMAN 1993), Bas1p appears specific for purine and histidine biosynthesis genes (ARNDT *et al.* 1987, DAIGNAN-FORNIER and FINK 1992, SPRINGER *et al.* 1996). Because all the genes known to be activated by Bas1p are also repressed by adenine, it is an appealing hypothesis that Bas1p has a direct role in regulating the purine biosynthetic pathway. There are several possibilities for how the availability of external adenine might be sensed. The purine bases themselves might be the signal. Alternatively, purine availability could affect transcription indirectly through a signalling cascade. Finally, Bas1p and/or Bas2p might be directly regulated by this signal.

As a first step toward answering these questions we have isolated mutants that constitutively express purine biosynthetic genes and are therefore candidate components of the signalling cascade that responds to environmental adenine. Here we report the isolation and characterization of these mutations and their cognate genes.

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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, guanine and hypoxanthine were used at a final concentration of 0.15 mM. The XGal synthetic medium (DANG *et al.* 1994) and the 5-fluoro-orotic acid (5-FOA) medium (BOEKE *et al.* 1984) were prepared using the methods

TABLE 1

Yeast strains used in this study

Strain name	Genotype	Source
PLY121	*MATα his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52*	P. LUNJDALL
PLY122	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52*	P. LUNJDALL
L3861	*MATα ade2 leu2-3,112 lys2-Δ201 ura3-52*	G. FINK
L3862	*MATα ade2 his3-Δ200 lys2-Δ201 ura3-52*	G. FINK
NC247-1B	*MAT***a** *ura3Δ fcy2Δ*	M. R. CHEVALLIER
W109-9C	*MAT***a** *ade2 trp1 ura3 his3 hpt1-27*	R. WOODS
AH215	*MAT***a** *leu2 his3*	M. KONRAD
AH215 *adk1*	*MAT***a** *leu2 his3 adk1::HIS3*	M. KONRAD
Y203	*MAT***a** *leu2-3,112 lys2 ura3-Δ100 ade2-1 his3 trp1 rnr3::RNR3-URA3-TRP1*	S. ELLEDGE
Y221	*MAT***a** *leu2-3,112 lys2 ura3-Δ100 ade2-1 his3 trp1 ctr6-68 rnr3::RNR3-URA3-TRP1* + pZZ13 *(HIS3)*	S. ELLEDGE
Y399	*MAT***a** *ade2 leu2-3,112 lys2-Δ201 his3-Δ200 ura3-52 bra9-1*	This work
Y531	*MATα leu2-3,112 lys2-Δ201 ura3-52 bra6-2*	This work
Y508	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 hpt1::URA3*	This work
Y511	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 apt1::URA3*	This work
Y520	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 aah1::URA3*	This work
Y548	*MATα leu2-3,112 lys2-Δ201 ura3-52 apt1::URA3*	This work
Y549	*MATα leu2-3,112 lys2-Δ201 ura3-52 apt1::URA3*	This work
Y550	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 apt1::URA3 aah1::URA3*	This work
Y551	*MAT***a** *leu2-3112 lys2-Δ201 ura3-52 apt1::URA3 aah1::URA3*	This work
Y552	*MATα leu2-3,112 lys2-Δ201 ura3-52 apt1::URA3 aah1::URA3*	This work
Y608	*MATα ade2 leu2-3,112 lys2-Δ201 ura3-52 ADE12::ADE12-LEU2*	This work
Y610	*MATα leu2-3,112 lys2-Δ201 his3-Δ200 ura3-52 ADE13::ADE13-LEU2*	This work

previously described. 5-fluorocytosine (5FC) was added at a final concentration of 0.1 mM to SC medium containing 0.03 mM uracil. Base analogues 8-azaadenine (8AA) and 8-azaguanine (8AG) were added to the media at a final concentration of 0.2 mg/ml.

Plasmids: P78, the plasmid carrying the *ADE5,7-URA3* chimera, was constructed by fusing the *ADE5,7* promoter and the first 28 codons of *ADE5,7* to the coding sequence of *URA3* (ALANI and KLECKNER 1987). For this purpose, the P4 plasmid carrying an *ADE5,7-lacZ* fusion (DAIGNAN-FORNIER and FINK 1992) in the vector YEp367R (MYERS *et al.* 1986) was digested with *Bam*HI and *Bgl*II and ligated to the *Bam*HI-*Bam*HI fragment carrying the *'URA3* gene from pNKY48 (ALANI and KLECKNER 1987).

***LacZ* fusions and βGal assays:** The *lacZ* fusions used in this study were constructed as follows. P2 and P115 have been previously described (DAIGNAN-FORNIER and FINK 1992). P2 is a plasmid carrying an *ADE2-lacZ* fusion in a 2μ *LEU2* vector YEp368R (MYERS *et al.* 1986). P115 is a plasmid carrying an *ADE1-lacZ* fusion in a 2μ *URA3* vector YEp356R (MYERS *et al.* 1986). Another *ADE1-lacZ* fusion was constructed in the course of this work using a two steps procedure. First a *Nsi*I-*Spe*I DNA fragment carrying the *ADE1* gene was cloned at the *Pst*I-*Xba*I sites of the pRS315 vector (SIKORSKI and HIETER 1989) generating plasmid P68. Second, a 1600-bp *Kpn*I-*Xba*I DNA fragment from P68 starting 900 bp upstream from the ATG initiation codon of the *ADE1* gene was cloned in YEp367 (MYERS *et al.* 1986).

βGal assays were performed as described by RUBY *et al.* (1984), with the exception of Table 8, assays that were performed by the method of KIPPERT (1995). In all cases, βGal units are defined as follows:

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

In each experiment, at least two independent βGal assays were performed, each assay was done on three independent transformants. Variation between assays in each experiment was <20%. Variations between experiments are due to the use of different spectrophotometers.

Mutagenesis: EMS mutagenesis was done on strains PLY121 and PLY122 transformed with the P78 plasmid. A 1-ml liquid culture of each strain grown overnight was centrifuged, washed twice with water and resuspended in 1 ml of EMS buffer (0.1 M KPO_4, pH 8). To each tube, 30 μl of EMS was added and vigorously mixed. After 1 h incubation at room temperature, the cells were pelleted and washed three times with 5% sodium thiosulfate. Finally cells were diluted in water and plated on YPD medium to estimate the loss of viability due to the mutagenesis. Comparison between treated and untreated cells allowed us to estimate at 60% the rate of survival after EMS treatment.

Integration of *LEU2* at the *ADE12* locus: An *Eco*RI-*Nsi*I fragment carrying the *ADE12* gene from plasmid P103 was cloned into an integrative *LEU2* vector named YIpLac128 (GIETZ and SUGINO 1988) linearized with *Eco*RI and *Pst*I. The resulting plasmid, named P659, was linearized at the *Pst*I site in the *ADE12* coding region and used to transform the L3861 strain. Tandem integration of the plasmid at the *ADE12* locus was verified by Southern blot on genomic DNA extracted from tranformants and cut with *Nsi*I. One of these transformants, named Y608, was used for linkage analysis.

Integration of *LEU2* at the *ADE13* locus: An *Eco*RI-*Bgl*II fragment carrying the *ADE13* gene was cloned into YIpLac128 (integrative *LEU2*, GIETZ and SUGINO 1988) linearized with *Eco*RI and *Bam*HI. The resulting plasmid, named P661, was linearized at the *Bam*HI site in the *ADE13* coding region and used to transform the PLY121 strain. Tandem integration of the plasmid at the *ADE13* locus was verified by Southern blot on genomic DNA extracted from tranformants and cut with *Bgl*II. One of these transformants, named Y610, was used for linkage analysis.

Disruption of the *HPT1* gene: Disruption of the *HPT1* gene was performed as follows. An *Eco*RV-*Eco*RV DNA fragment carrying the *YDR399w* open reading frame (ORF) from chromosome IV was inserted in pUC18 (YANISCH-PERRON *et al.* 1985) linearized at the unique *Sma*I site. The resulting plasmid, named P385, was deleted for its internal *Bgl*II-*Bgl*II DNA fragment thus removing the promoter region and the 5′ half of the coding region (this deletion does not affect the upstream ORF named *YDR398w*). The deleted fragment was replaced by a *Bam*HI-*Bam*HI fragment carrying the *URA3* gene from plasmid YDpURA3 (BERBEN *et al.* 1991). The resulting plasmid, named P399, was digested with *Nsi*I and used to transform the PLY122 strain. Ura$^+$ transformants resulting from one-step gene disruption at the *HPT1* locus were obtained and verified by Southern blot (data not shown).

Disruption of the *AAH1* gene: For disruption of the *AAH1* gene, a 1300-bp *Eco*RI-*Sph*I fragment carrying the *AAH1* gene from pCG22 (DEELEY 1992) was cloned in YIpLac128 (GIETZ and SUGINO 1988). This plasmid, named P389, was deleted for its *Pst*I-*Xba*I 116-bp fragment that is located 65 bp downstream of the ATG initiation codon of the *AAH1* ORF, and this fragment was replaced by a *Nsi*I-*Spe*I fragment carrying the *URA3* gene from YEp24. The resulting plasmid, named P456, was digested with both *Ssp*I and *Sph*I and used to transform the PLY122 strain. Disruption was verified by Southern (data not shown).

Disruption of the *APT1* gene: A 1450-bp *Eco*RV-*Kpn*I fragment carrying the *APT1* gene from pCG42 (DEELEY 1992) was cloned in pBluescript KS digested with *Eco*RV and *Kpn*I. The resulting plasmid, named P393, was deleted for its internal *Hin*dIII fragment containing most of the *APT1* ORF. The deleted fragment was replaced by the 1.1-kb *Hin*dIII fragment carrying the *URA3* gene. The plasmid carrying the *APT1::URA3* construct (P397) was digested with *Eco*RV and *Kpn*I and used to transform the yeast strain PLY122. Disruption was verified by Southern (data not shown).

Adenylosuccinate lyase enzymatic assay: Adenylosuccinate lyase activity was measured according to the method of WOODWARD (1978). Briefly, yeast strains were grown in 50 ml of SD medium to an OD_{600} of 0.6 ± 0.1. Cells were harvested, washed with breaking buffer (0.05 M TrisCl pH 8, 20% glycerol and 1 mM DTT) and resuspended in 0.250 ml breaking buffer. Phenylmethyl sulfonyl fluoride (PMSF) was added to a final concentration of 2 mM and the cells were then broken with glass beads by vortexing four times for 30 sec in the cold. After addition of 0.250 ml of breaking buffer, glass beads and unbroken cells were pelleted in a microfuge for 5 min and either 50 or 100 μl of the supernatant were used for the enzymatic assay. The assay was done in 50 mM TrisCl pH 8, 48 μM adenylosuccinate monophosphate (AMPS) in a final volume of 1 ml. Conversion of AMPS to AMP by adenylosuccinate lyase was followed as the decrease of absorbance (10.7/nmol/ml) at 280 nm. The specific activities are expressed as nanomoles of AMPS consumed per min per mg of protein. Protein concentration was determined using the Bio-Rad Protein Micro Assay System, with crystalline bovine serum albumine serving as the reference standard.

RESULTS

In this section we will first describe isolation of mutants that constitutively express adenine biosynthetic genes, then we will present our analysis of the mutant phenotypes and the results of the complementation analysis. Finally, characterization of several genes involved in the signalling cascade will be shown.

Isolation of *bra* mutants: We have used a *ADE5,7-URA3* translational fusion to select for mutations that affect repression of *ADE* genes in the presence of adenine. A fusion between the *ADE5,7* promoter and the *URA3* coding sequence (ALANI and KLECKNER 1987) was constructed on a 2μ yeast expression plasmid. Expression of *URA3* in this construct is under the control of *ADE5,7* regulatory elements, *i.e.*, activated in the presence of Bas1p and Bas2p and repressed by adenine. A *ura3* strain containing this plasmid, grows very slowly on medium lacking uracil and containing adenine (repression conditions). In the absence of adenine the growth is faster but still slower than in the presence of uracil, indicating that the OMP decarboxylase activity provided by the *ADE5,7-URA3* fusion is not optimal even under derepression conditions. The growth rate difference between repression and derepression conditions in the absence of uracil makes it possible to identify constitutive mutants as rapidly growing colonies above the background of slowly growing colonies.

The *ADE5,7-URA3* fusion carried on a plasmid was introduced in **a** and α isogenic wild-type strains, PLY121 and PLY122. These transformed strains were mutagenized with ethyl methanesulfonate to 60% survival and plated at low density (~2000 cfu/plate) on SC −leu −ura + ade. After 4–5 days, 84 mutants (42 in each mating type) that grew faster than the wild-type strain under repression conditions were selected for further analysis. Mutants named 101–142 were isolated from PLY121 and mutants 201–242 from PLY122.

We anticipated two genetic events that would lead to a fast growing phenotype and that would not be due to changes in the regulation pathway for purine biosynthesis: gene conversion at the *ura3* locus or *cis*-acting mutations in the *ADE5,7-URA3* reporter. To eliminate these two classes of mutations, we independently tested expression from an *ADE1-lacZ* fusion. *URA3* gene conversions or promoter mutations in the *ADE5,7* fusion would be expected to show normal regulation of the *ADE1-lacZ* reporter. *Bona fide* transacting mutations should show constitutive expression of this reporter. This second reporter also allowed us to measure the degree of the derepression in the mutant strains.

All the 84 candidate constitutive mutants were grown on medium containing 5-fluoroorotate (5FOA) (BOEKE *et al.* 1984) to cure the *ADE5,7-URA3* plasmid. These strains were then retransformed with a plasmid carrying an *ADE1-lacZ* fusion (named P115). β-galactosidase (β-Gal) activity was then determined under repression and derepression conditions. Thirty-nine mutants were not studied further because they either did not grow on 5FOA or displayed normal regulation of *ADE1-lacZ* expression. The remaining mutants were called *bra* for bypass of repression by adenine.

The *bra* mutants fall into three phenotypic classes (see Figure 1 for examples of each class). The class 1

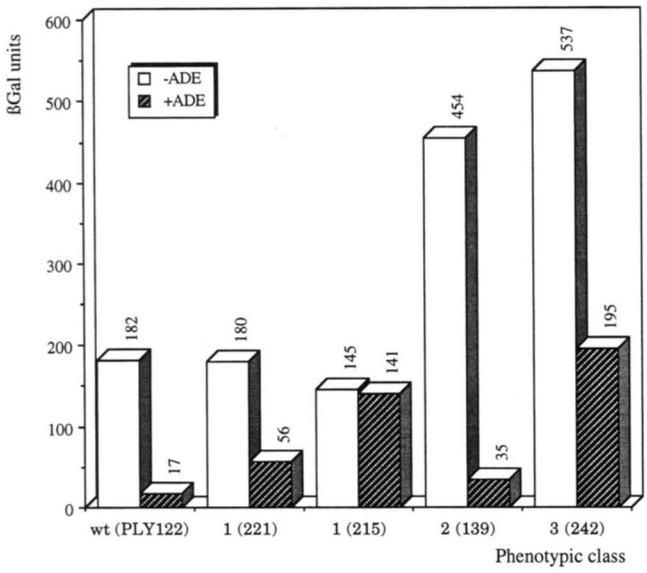

FIGURE 1.—Examples of the three classes of *bra* mutants. Constitutive expression was tested using an *ADE1-lacZ* fusion. Vertical bars represent βGal units under derepression and repression conditions for the wild-type strain (wt) and the three different phenotypic classes numbered 1–3 below the line. For each example, the mutant name is indicated between parentheses.

mutants express the fusion at the wild-type level under derepression conditions. Under repression conditions (in the presence of adenine), expression of the fusion is increased in the mutant relative to the wild-type strain. The repression factor, defined as the ratio of fusion expression in the absence and in the presence of adenine, varied from 0.9 to 5.9 in this class of mutants compared to 7.6 and 10.7 in the two wild-type strains. A total of 19 mutants fall into class 1. The class 2 mutants express more fusion protein than wild type under both repression and derepression conditions. For the two mutants in this class the repression factor is the same as in the wild-type control. Class 3 mutants express more βGal activity than wild-type under both repression and derepression conditions but the repression factor is much lower than in wild-type cells (1.1 to 5.8 compared to 7.6 and 10.7 in the two wild-type strains). The size of this class is 20. Finally, four mutants could not be placed into classes either because they revert with a very high frequency (mutants 134 and 224) or are unable to grow in the absence of adenine (mutants 115 and 241) and could therefore not be tested for derepression.

A similar analysis was performed on five *bra* mutants with an *ADE2-lacZ* fusion (Table 2). These results show that the phenotypic classes are not specific for the *ADE1-lacZ* fusion. We also tested whether the *bra* mutants require Bas1p to express their derepression phenotype. The same five mutants were tested for their ability to express an *ADE2-lacZ* fusion with no Bas1p binding site in its promoter (DAIGNAN-FORNIER and FINK 1992). In these five *bra* mutants, expression of the mutated *ADE2-lacZ* fusion was as low as in the wild-type control strain, showing that Bas1p is absolutely required for the expression of the constitutive phenotype of these mutants (Table 2).

TABLE 2

Expression of *ADE1-LacZ*, *ADE2-LacZ* and a mutated version of *ADE2-LacZ* deprived of its two Bas1p binding sites in the wild-type strain PLY122 and five *bra* mutants

	βGal units					
	ADE1-LacZ		*ADE2-LacZ*		*mutADE2-LacZ*	
Strain	+ade	−ade	+ade	−ade	+ade	−ade
PLY122	17	182	52	157	15	13
128	106	150	230	209	17	16
131	136	158	175	195	15	18
216	142	139	177	176	19	17
217	661	757	598	560	6	5
220	245	208	174	182	14	13

Additional phenotypes conferred by the *bra* mutations: All the mutants were tested for growth defects, eight of them exhibit a slower growth at 15°. It has not been shown yet whether this phenotype is linked to the derepression of adenine biosynthetic genes, but the fact that several of these mutants belong to common complementation groups (see below) is consistent with the idea that the cold-sensitivity and derepression are the result of the same mutation. As stated above, two mutants (115 and 241) are complete adenine auxotrophs and another mutant (217) is a partial auxotroph.

Mutations in genes encoding enzymes in purine salvage pathways might be expected to produce a *bra* derepression phenotype. Such mutations should be unable to convert base analogues into the the toxic derivatives that act *in vivo* and are therefore likely to confer resistance to these drugs. Possible resistance of the *bra* mutants to three such analogues, 8-azaadenine (8AA), 8-azaguanine (8AG) and 5-fluorocytosine (5FC), was therefore tested by a petri plate growth assay. Several of the *bra* mutants do confer resistance to one or more of these drugs (Table 3).

The *bra* mutants fall into more than seven complementation groups: Each of the *bra* mutant was crossed to an isogenic wild-type strain. The phenotype of the heterozygous diploids was then scored using the growth assay for expression of the *ADE5,7-URA3* fusion. Thirty mutants were found to be fully recessive and 15 were dominant or semidominant. Pairwise crosses of the recessive mutants from opposite mating types were made and a complementation analysis was performed. The partially dominant mutants were also crossed to opposite mating type mutants and growth of these diploids in the absence of uracil and in the presence of adenine

TABLE 3

Phenotypical analysis of the 20 mutants belonging to complementation groups *bra1–bra7*

Mutant[a]	Class	βGal units (*ADE1-lacZ*) +ade	βGal units (*ADE1-lacZ*) −ade	Repression factor	Additional phenotypes[b]
PLY122 (wt)		17	182	10.7	
bra1-1 (115)	NA[c]				Ade$^-$
bra1-2 (213)	3	109	299	2.7	
bra1-3 (231)	3	109	410	3.8	
bra2-1 (118)	3	56	291	5.2	cs, SD, 8AA^R
bra2-2 (124)	3	172	408	2.4	cs, 8AA^R
bra2-3 (211)	3	197	413	2.1	8AA^R
bra3-1 (129)	1	61	171	2.8	8AA^R, 8AG^R
bra3-2 (130)	1	114	186	1.6	8AA^R, 8AG^R
bra3-3 (220)	1	245	208	0.8	8AA^R, 8AG^R
bra3-4 (239)	1	102	162	1.6	8AA^R, 8AG^R
bra4-1 (132)	2	68	560	8.2	cs, 8AA^R
bra4-2 (242)	3	195	537	2.8	cs, 8AA^R
bra5-1 (109)	1	26	153	5.9	
bra5-2 (206)	3	278	479	1.7	cs, SD, 8AA^R
bra5-3 (208)	3	253	471	1.9	cs, SD, 8AA^R
bra6-1 (134)	[d]				
bra6-2 (216)	1	142	139	1.0	8AG^R
bra6-3 (224)	[d]				
bra7-1 (119)	1	89	143	1.6	5FC^R, 8AA^R, 8AG^R
bra7-2 (232)	1	142	204	1.4	5FC^R, 8AA^R, 8AG^R

[a] wt, stands for wild-type control, numbers inside brackets indicate original mutant name.

[b] cs, SD, 8AA^R, 5FC^R and 8AG^R stand, respectively, for cold-sensitive, semi-dominant, resistant to 8-azaadenine, resistant to 5-fluorocytosine and resistant to 8-azaguanine. Assignment of semi-dominant mutants to complementation groups is uncertain (see text for details).

[c] NA, not applicable.

[d] These two mutant strains belonging to the *bra6* complementation group revert with high frequency and were not tested for additional phenotypes.

was estimated by comparison to the wild-type isogenic cross. Since these partially dominant mutants may not be due to loss-of-function mutations, assignments of these mutants to a given complementation group are uncertain. As shown in Table 3, 20 *bra* mutants define at least seven complementation groups. The remaining mutants complement all mutants of opposite mating type. In most cases, the mutants in a given complementation group show similar additional phenotypes and fall in the same phenotypic class (see previous section and Table 3).

***BRA7* is *FCY2*:** One possible mechanism by which some of our mutants fail to repress gene expression in response to adenine is that these mutants cannot take up adenine from the media. We have used a drug resistance test previously described (CHEVALLIER *et al.* 1975) to identify such mutants. This test is based on the fact that mutations in the gene *FCY2*, encoding the purine-cytosine permease, lead to resistance to a toxic cytosine analogue, 5-fluorocytosine (5FC). The *bra* mutants were therefore tested for growth on medium supplemented with 5FC.

Two mutants (119 and 232), belonging to the same complementation group (*bra7*), are resistant to 5FC. Both mutants are also resistant to other purine base analogues, probably because the transport of these drugs is diminished in these mutants. When *bra7-1* was crossed to a wild-type strain, the absence of repression by adenine (followed by the growth assay for the expression of the *ADE5,7-URA3* fusion) segregated as a mutation in a single nuclear gene (2:2 in 16 tetrads).

Complementation analysis suggested that *bra7-2* is allelic to *FCY2*. A centromeric plasmid carrying the *FCY2* gene (pRFF2 kindly provided by M. R. CHEVALLIER) restores normal regulation of an *ADE1-lacZ* fusion when introduced into *bra7-2* (data not shown). Also we found that a strain containing a deletion of *FCY2* fails to complement a *bra7-1* strain. The adenine repression factor in a *fcy2/BRA7* diploid is 7.0 and falls to 2.9 in a *fcy2/bra7* isogenic diploid. Furthermore, the latter diploid is resistant to 5FC but the former is sensitive. Finally, both diploids were sporulated and after tetrad dissection the resistance to 5FC segregated 2:2 in the *fcy2/BRA7* diploid (eight tetrads) while only 5FC^R spores

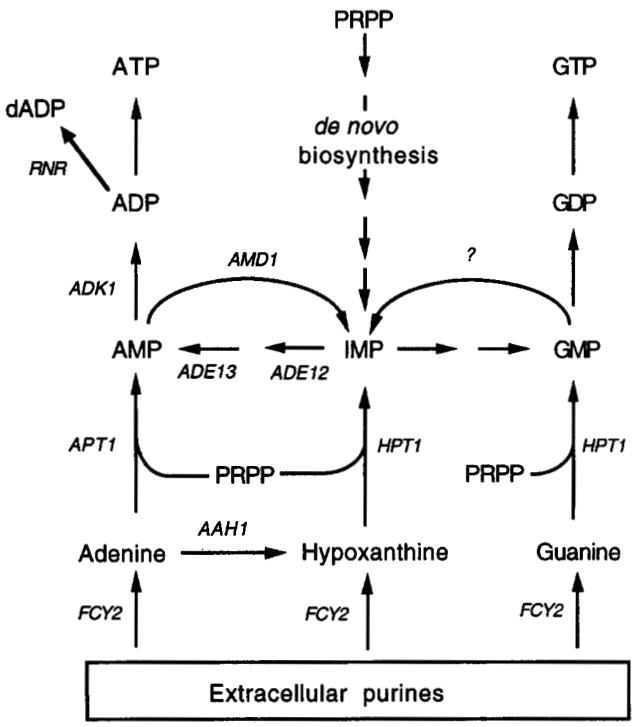

FIGURE 2.—Schematic representation of purine interconversion in yeast. The following abbreviations are used: PRPP, 5-phosphoribosyl-1-pyrophosphate; IMP, inosine 5′-monophosphate. Genes names are indicated in italic and encode the following enzymatic activities: *AAH1*, adenine deaminase; *ADE12*, adenylosuccinate synthetase; *ADE13*, adenylosuccinate lyase; *ADK1*, AMP kinase; *AMD1*, AMP deaminase; *APT1*, adenine phosphoribosyl transferase; *FCY2*, purine cytosine permease; *HPT1*, hypoxanthine guanine phosphoribosyl transferase; *RNR*, ribonucleotide reductase. The ? symbol represents possible GMP reductase activity discussed in the text. For simplification purpose nucleosides are not represented.

were recovered in the nine tetrads dissected from the *fcy2/bra7* diploid. Cosegregation of 5FC resistance and constitutive expression of *ADE1-lacZ* expression was confirmed, establishing linkage between *fcy2* and *bra7*. From these data we infer that the purine cytosine permease is required for adenine repression, most likely because the transduction pathway monitors the intracellular concentration of adenine or a derivative.

***BRA9* is *ADE12*:** Two mutants, *bra1-1* (115) and *bra9-1* (241), are adenine auxotrophs. Both were tested for complementation by strains containing mutations that affect steps in the *de novo* pathway for adenine biosynthesis (*ade1–ade9*); both mutants complement all the tested mutations. *bra1-1* and *bra9-1* do not grow on a medium supplemented with hypoxanthine, a base that is converted into IMP and can therefore rescue all the mutants affecting the *de novo* pathway leading to synthesis of IMP (see Figure 2). *bra1-1* and *bra9-1* therefore appear to be "adenine-specific." Two mutants with similar adenine-specific phenotypes, *ade12* and *ade13*, have been described (DORFMAN 1969). Additional phenotypes found in *ade12* mutants (DORFMAN 1969) and in *bra1-1* and *bra9-1* are as follows: (1) poor growth at low concentrations of adenine even on rich medium, (2) spores carrying the mutation germinate poorly, (3) germination defect that can be rescued by a mutation upstream in the pathway.

Because of the germination defect of the adenine-specific mutants, it was not possible to study the segregation of *bra9-1*. To bypass this problem, the *bra9-1* mutant was crossed to an isogenic wild-type strain (L3862) carrying an *ade2* mutation, since the germination defect of *ade12* strains was rescued by mutations upstream in the pathway. A spore carrying both *ade2* and *bra9-1* mutations was selected on the basis of its red color (characteristic of *ade2* mutants) and growth on adenine and not hypoxanthine due to the *bra9-1* mutation. This strain was then crossed to an isogenic wild-type strain carrying an *ade2* mutation (Y608); the resulting diploid is homozygous for *ade2* and heterozygous for *bra9-1*. Fourteen tetrads from this cross were analyzed. We observed 2:2 segregation for lack of growth on hypoxanthine supplemented medium and for derepressed expression of the *ADE1-LacZ* fusion. Both phenotypes cosegregate.

To isolate the *BRA9* gene, the *bra9-1* original mutant was transformed with a yeast genomic library carried on a centromeric vector. Two plasmids able to complement the adenine requirement of *bra9-1* were isolated. These plasmids were shown to carry overlapping sequences that hybridize to chromosome XIV (data not shown). The plasmid P103, able to complement the *bra9-1* auxotrophy, was recently also shown to carry the *ADE12* gene (GALLERT *et al.* 1996). This conclusion is based on several lines of evidence: (1) it can specifically suppress the growth defect of a *purA* mutant in *E. coli* (*purA* is the gene encoding adenylosuccinate synthetase in *E. coli*), (2) the P103 plasmid contains a sequence encoding a polypeptide highly similar to adenylosuccinate synthetases from other organisms, (3) this sequence maps physically very close to the *ade12* locus.

The conclusion that *bra9-1* and *ade12* are mutations in the same gene is further supported by the fact that the *bra9-1* mutation cannot complement the auxotrophic phenotype of an *ade12* mutant. Both mutations also lead to an absence of adenylosuccinate synthetase activity *in vitro* (GALLERT *et al.* 1996). Finally, linkage between *bra9* and the cloned *ADE12* gene was also demonstrated. The *LEU2* marker was integrated at the *ADE12* locus by transformation in an *ade2* mutant strain (see MATERIALS AND METHODS). The resulting strain, named Y608, was crossed to the *ade2* Y399 strain carrying the *bra9-1* mutation. In 14 tetrads only parental ditypes were observed (all the Leu^+ spores were Ade^+ and all the Leu^- spores were Ade^-). Together, these results show that *bra9-1* is a mutation in the *ADE12* gene encoding adenylosuccinate synthetase.

***bra1* mutants affect adenylosuccinate lyase activ-**

ity: The second mutant showing an adenine-specific requirement is *bra1-1*. Interestingly, the *bra1-2* and *bra1-3* mutants are not adenine auxotrophs, demonstrating that the constitutive expression phenotype and the adenine requirement can be separated. The Bra$^-$ phenotype (monitored with an *ADE1-lacZ* fusion) of the *bra1-3* mutant was shown to segregate 2:2 in a cross with an isogenic wild-type strain (19 tetrads). The *BRA1* gene was cloned by complementation of the adenine requirement of a *bra1-1* strain. Two plasmids carrying overlapping genomic inserts were isolated. As expected, the *BRA1* gene also complemented *bra1-2* derepression phenotype.

Surprisingly a third mutation, *bra8-1* (mutant 217), was also complemented by *BRA1* both for derepression and for a slight adenine requirement that cannot be rescued by hypoxanthine. When *bra8-1* was crossed to an isogenic wild-type strain (PLY121), the subtle adenine requirement was shown to segregate 2:2 in 10 tetrads. Furthermore, using an *ADE2-lacZ* reporter, the adenine requirement of *bra8-1* was found to be linked to the derepression phenotype. Since *bra8-1* and *bra1-1* are fully recessive and fully complement for derepression, these two mutations were placed into different complementation groups, but the complementation of both mutants by the same plasmid strongly suggest that they are complementing alleles of the same gene. Linkage between *bra1* and *bra8* was studied by crossing *bra1-2* and *bra8-1* strains, the resulting diploid was sporulated and the spores were analyzed for adenine repression using an *ADE1-lacZ* fusion. All the tetrads (20) contain four spores showing a Bra$^-$ phenotype demonstrating that *bra1* and *bra8* are tightly linked. This result is not surprising since intragenic complementation has been described previously at homologous loci in other organisms (WOODWARD *et al.* 1958; FOLEY *et al.* 1965).

The structure of the *BRA1* genomic locus was further characterized. It contains four internal *Bam*HI fragments (between 1.5 and 2.5 kb, see Figure 3). Deletion of all four fragments abolished complementation. None of the four fragments alone complemented *bra1-1,* suggesting that one of the *Bam*HI site was in *BRA1* (see Figure 3). The ends of the *Bam*HI fragments were therefore sequenced and two of them were found to be in an open reading frame (YLR359w) encoding a polypeptide highly similar to adenylosuccinate lyase (ASL) in other organisms (see Figure 4). Of note, a histidine residue that is part of the *B. subtilis* enzyme active site (residue 141, LEE *et al.* 1997) is conserved in the yeast sequence (at position 134). ASL catalyzes two steps in the purine biosynthesis pathway, one in the *de novo* biosynthesis pathway and one in the interconversion of IMP into AMP. Adenylosuccinate lyase activity was previously shown to be abolished by *ade13* mutations (DORFMAN 1969). The *ade13* locus has not been mapped.

A 2.9-kb *Eco*RI fragment carrying only the *YLR359w* ORF (see Figure 3) was subcloned in a *LEU2* CEN vector and shown to be able to restore adenine prototrophy to the *bra1-1* and *bra8-1* mutants. Furthermore, the same plasmid can complement the derepression phenotype of *bra1-2* and *bra1-3* mutant strains (data not shown). Linkage between *bra1* and the *ADE13* gene was established as follows: the *LEU2* marker was integrated at the *ADE13* locus by transformation (see MATERIALS AND METHODS for details), the resulting strain (Y610) was then crossed to the *bra1-3* mutant and expression of an *ADE1-lacZ* fusion in 19 tetrads from this cross was determined. Constitutive expression of the fusion in the presence of adenine was found in all the Leu$^-$ spores and in none of the Leu$^+$ spores. *bra1* and *ADE13* are therefore tightly linked. It is very likely that *bra1* and *bra8* are allelic to the previously described *ade13* locus (DORFMAN, 1969) but linkage analysis could not be performed because the original *ade13* mutants have been lost.

To further test if the *bra1* and *bra8* mutants are mutations in the structural gene for adenylosuccinate lyase, enzymatic activity was measured in these mutant strains. We found that enzymatic activity is low in all the *bra1* mutants and in the *bra8* mutant (Table 4). It is not affected in the *bra9* mutant, which is not complemented by the plasmid carrying the ASL encoding gene. Both *bra1* and *bra8* mutants therefore have decreased adenylosuccinate lyase activity, consistent with our genetic analysis. Of note, the lowest ASL activity was observed in the *bra1-1* mutant; *bra1-1* is the allele that confers an adenine auxotrophy. The prototrophic alleles lead to decreased enzymatic activity but apparently a low level of activity is sufficient to sustain growth in the absence of adenine. This demonstrates that mutations at this locus causing derepression of the purine pathway can cause either adenine auxotrophy or not.

***BRA6* is *HPT1*, the gene encoding hypoxanthine-guanine phosphoribosyl transferase:** The *bra6* complementation group contains three alleles. Two of the *bra6* alleles (134 and 224) are difficult to study because they revert at a high frequency. Further studies on this complementation group were therefore done with *bra6-2*. This mutant belongs to the first phenotypic class (Table 3). The *bra6-2* mutant was crossed to an isogenic wild-type strain (PLY121) and the resulting diploid was sporulated. The derepression phenotype associated to the *bra* mutation was followed using an *ADE1-lacZ* fusion and shown to segregate 2:2 in 11 tetrads (data not shown).

BRA6 was cloned by complementation of *bra6-2*. A genomic library carried on a *LEU2* centromeric vector was introduced into *bra6-2* and complementing clones were identified by the ability to restore wild-type regulation of the *ADE1-lacZ* fusion in the presence of adenine. Two complementing plasmids (P132 and P133) were

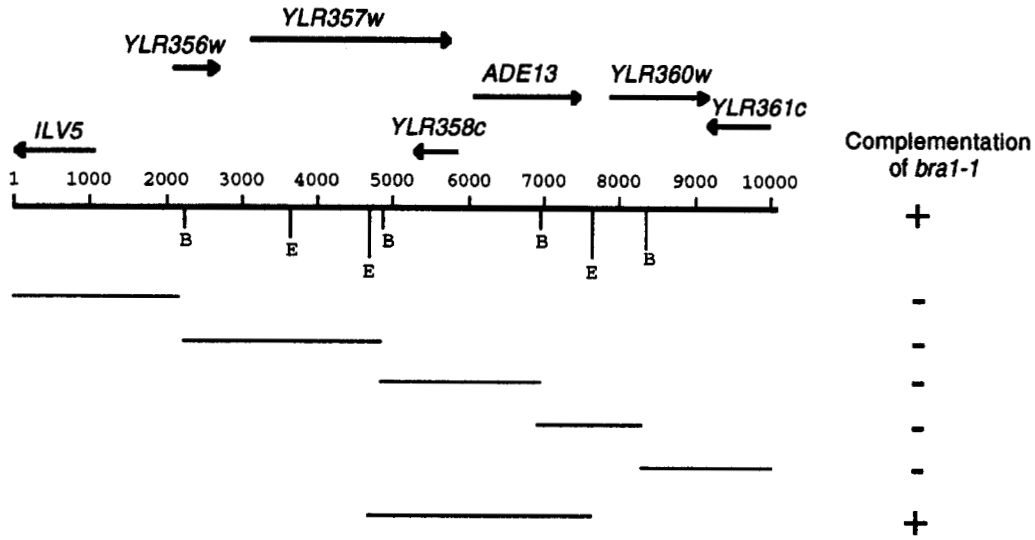

FIGURE 3.—Schematic physical map of the yeast DNA insert carried by P127 plasmid that complements the *bra1-1* auxotrophy for adenine. Numbers over the line refer to the size of the DNA fragment in base pairs. E and B stand for *Eco*RI and *Bam*HI restriction sites, respectively. ORFs longer than 100 codons deduced from the complete nucleotide sequence of chromosome XII are represented as arrows on the top of the drawing. Restriction fragments used for subcloning experiments are drawn at the bottom of the drawing with complementation result presented on the right part of the figure.

isolated. One plasmid (P133) was used for physical mapping. Hybridization to separated yeast chromosomes demonstrated that the P133 insert is derived from chromosome IV. This result was confirmed by hybridization to an ordered lambda library (kindly provided by L. RILES and M. OLSON) revealing two spots corresponding to two overlapping clones mapping between *ade8* and *snf1*. The chromosome IV sequences from the yeast genome sequence allowed us to precisely position the P132 and P133 insert sequences. As shown in Figure 5, the two plasmids contain overlapping sequences, the common part of which contains four ORFs. Further subcloning (see Figure 5) established that *YDR399w* (plasmid P386) is the locus that complements the Bra phenotype (Table 5A). This ORF encodes a polypeptide presenting some similarities with hypoxanthine phosphoribosyl transferases (HPRT) from several organisms (see Figure 6). One of the most conserved regions (residues 103 to 119 in the yeast sequence) is a potential PRPP binding site as defined by HERSHEY and TAYLOR (1986). Mutations named *hpt1* had been previously characterized by WOODS and coworkers that lead to the loss of HPRT activity and resistance to a base analogue named 8-azaguanine (8AG) (WOODS *et al.* 1983). Indeed, the *bra6-2* mutant is specifically resistant to 8AG, suggesting that *bra6* and *hpt1* could be the same locus (Table 3).

The following experiments were performed to confirm that *bra6-2* is allelic to *hpt1*. First, we took advantage of a strain (W109-9C) mutated at the *hpt1* and *ade2* loci. Such a double mutant cannot perform the *de novo* synthesis of purines and cannot use hypoxanthine as a purine source. However, this double mutant can grow when adenine is provided as a purine source because adenine phosphoribosyl transferase (APRT) can convert adenine to AMP, which is then converted to IMP by AMP deaminase (see Figure 2). Therefore this mutant will grow on adenine but not on hypoxanthine as a purine source. The P386 plasmid carrying only the *YDR399w* ORF (see Figure 5) and a control plasmid (pRS316) were introduced into the W109-9C strain. Growth of the transformants was then monitored on minimal medium with either adenine or hypoxanthine as a purine source. By contrast with the control plasmid, P386 supported growth on media containing hypoxanthine as a purine source. Because the *YDR399w* ORF carried on a centromeric vector complements the *hpt1* mutation, this ORF was renamed *HPT1*.

The *HPT1* gene was disrupted in the PLY122 strain (MATERIALS AND METHODS). Isogenic wild-type and disrupted strains were then transformed with plasmid P473 carrying a *ADE1-lacZ* fusion. In the *hpt1::URA3* strain (Y508) the fusions are derepressed in the presence of adenine (Table 5B) and are resistant to 8AG. Linkage analysis also confirmed that *bra6* and *hpt1* are the same locus. A *bra6-2* strain (Y531) was crossed to the *hpt1::URA3* strain and the diploids were sporulated. In 10 tetrads the resistance to 8AG segregated 4:0 ($8AG^R$:$8AG^S$). Furthermore, with the *ADE1-lacZ* fusion introduced into the spores from five tetrads, we found that all the meiotic progeny from this cross showed the derepression phenotype. The factor of repression by adenine in the mutant spores varied from 0.9 to 2.5, while it was 4.1 in the wild-type control. As a final test of the idea that *bra6* and *hpt1* are allelic, the *bra6-2* mutant strain Y531 and the isogenic wild-type strain

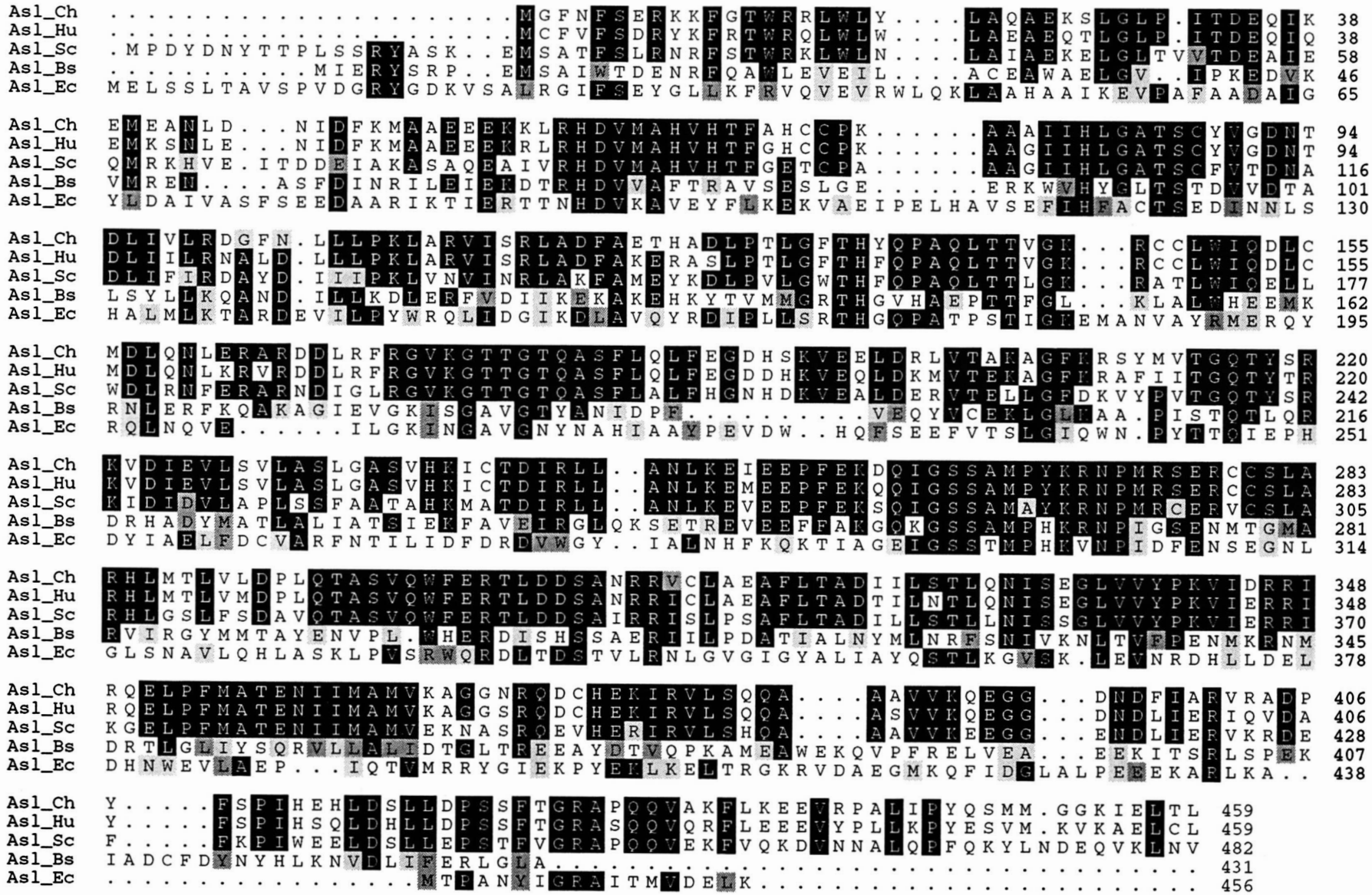

FIGURE 4.—Comparison of adenylosuccinate lyase sequences from various organisms. The amino acid sequence deduced from the nucleotide sequence of the *ADE13* gene (Asl_Sc) was compared with adenylosuccinate lyase sequences from chicken (Ch), human (H), *B. subtilis* (Bs) and *E. coli* (Ec). Identies and similarities are highlighted in black and gray boxes, respectively. Periods indicate the gaps created for alignment.

TABLE 4

Adenylosuccinate lyase activity in the *bra1, bra8, bra9* mutants and isogenic PLY121 and PLY122 wild-type strains

Strain	Activity (nmol AMPS/min/mg prot)
PLY121	10.0 ± 2.0
bra1-1	0.2 ± 0.2
PLY122	13.3 ± 2.6
bra1-2	1.1 ± 0.4
bra1-3	1.2 ± 0.5
bra8-1	2.1 ± 0.3
bra9-1	18.0 ± 0.8

Activity is the average of three assays. See MATERIALS AND METHODS for details.

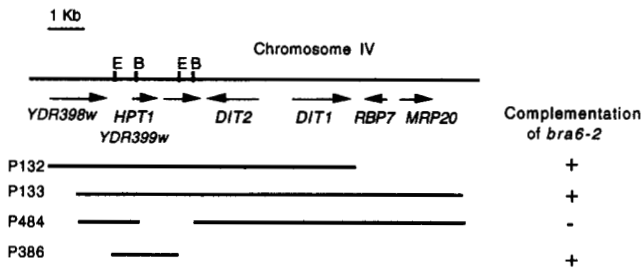

FIGURE 5.—Physical map of the chromosome IV region carrying the *HPT1* gene. E and B stand for *Eco*RI and *Bam*HI restriction sites, respectively. ORFs deduced from the nucleotide sequence are represented as arrows below the line. Subcloning strategy and results of complementation are presented at the bottom of the figure.

PLY121 were crossed to the previously characterized *hpt1* mutant from WOODS and coworkers (W109-9C). Although the heterozygous *HPT1/hpt1* diploid was sensitive to 8AG, the *bra6* × *hpt1* diploid is resistant (data not shown). Both diploids were tranformed with the P115 plasmid carrying an *ADE1-lacZ* fusion and the repression by adenine was estimated by measuring βGal activity in the presence or absence of adenine. Results presented in Table 5C clearly show that *hpt1* cannot complement *bra6-2* for derepression. The *bra6* × *hpt1* diploid was sporulated and segregation of resistance to 8AG was monitored in 14 tetrads. All of the spores from this cross were resistant to 8AG, demonstrating that *bra6* and *hpt1* loci are tightly linked. In sum, we conclude that *bra6* and *hpt1* are the same locus and that they encode yeast HPRT.

Role of the *APT1* and *AAH1* genes in the process of repression by adenine: Once inside the cell, adenine can take two different metabolic routes (see Figure 2). It can be metabolized into AMP by APRT (encoded by the *APT1* gene, ALFONZO *et al.* 1995). Alternatively, it can be deaminated to hypoxanthine by the adenine deaminase (encoded by the *AAH1* gene, WOODS *et al.* 1984, DEELEY 1992), and then transformed into IMP by HPRT (encoded by the *HPT1* gene, WOODS *et al.* 1983 and this work). Our finding that *bra6* is allelic to *hpt1* demonstrated that the "HPRT route" plays an important role in the process of adenine repression. To further evaluate the contributions of these two pathways, we constructed isogenic strains with disruptions of *apt1, aah1* and *hpt1* (see MATERIALS AND METHODS). Adenine regulation was analyzed in these strains (termed Y511, Y520 and Y508, respectively). We also tested two other purine bases, hypoxanthine and guanine, for effects on transcriptional repression of the *ADE1-lacZ* gene fusion. Several conclusions can be drawn from the results presented in Table 6A. First, adenine and hypoxanthine cause similar levels of repression but guanine causes only partial repression. It is noteworthy that the effects of adenine, hypoxanthine and guanine on transcriptional repression are not additive (data not shown), suggesting that they act through the same signalling pathway. Second, the *apt1* mutation does not affect repression by adenine, suggesting that the adenine repression signal could be carried by a metabolite in the HPRT route. If this is correct, *aah1* mutations should have the same effect on derepression as *hpt1* mutations. Surprisingly although an *hpt1* null allele leads to derepression, mutation of the *aah1* locus has no effect on adenine repression. We interpret this observation as follows. In wild-type strains most of the available adenine is metabolized to hypoxanthine by adenine deaminase; however, in the *hpt1* mutant strain, hypoxanthine cannot be further metabolized and therefore does not activate a repression signal. In the *aah1* mutant, the adenine normally deaminated by Aah1p is available for utilization by Apt1p, thus allowing synthesis of the factor that activates repression.

By this hypothesis, adenine that is not used in one route is used in the other. A prediction of this model is that a double *aah1 apt1* mutant should be fully derepressed. The desired double mutant was isolated from a cross between *apt1::URA3* (Y548 or Y549) and *aah1::URA3* (Y520), haploid strains. Three such double mutant spores (Y550, Y551 and Y552) were isolated and the presence of the double disruption was confirmed by Southern blot analysis (data not shown). The *ADE1-lacZ* reporter was introduced into these strains, and the effect of adenine, hypoxanthine and guanine on expression of the fusion in the transformed strains was determined (Table 6B). As predicted, in the double *aah1 apt1* mutant regulation by adenine is abolished while regulation by hypoxanthine is unaffected. Another prediction is that overexpression of *APT1* should increase the flux of adenine used for synthesis of AMP through APRT and should therefore at least partially bypass the deregulation in the *hpt1* mutant. The *ADE1-lacZ* reporter and a multicopy plasmid carrying either the *APT1* gene (pCG3, DEELEY 1992) were introduced into *bra6-2* mutant strain. Results presented in Table 7 clearly show that overexpression of *APT1* abolishes the derepression phenotype of the *hpt1* mutation, therefore

TABLE 5

Expression of *ADE-LacZ* fusions in different *HPT1* genetic backgrounds

Strain	Relevant genotype	Plasmids		βGal activity			
		LacZ fusion	Other	+ade	−ade	RF[a]	8AG[b]
A.							
PLY122	(*BRA6*)	P2 (*ADE2-LacZ*)	pRS316 (control)	27	80	3.0	S[c]
PLY122	(*BRA6*)	P2 (*ADE2-LacZ*)	P386 (*HPT1* CEN)	28	83	3.0	S
216	(*bra6-2*)	P2 (*ADE2-LacZ*)	pRS316 (control)	66	80	1.2	R
216	(*bra6-2*)	P2 (*ADE2-LacZ*)	P386 (*HPT1* CEN)	28	77	2.8	S
B.							
PLY122	(*HPT1*)	P473 (*ADE1-LacZ*)	—	28	169	6.0	S
Y508	(*hpt1::URA3*)	P473 (*ADE1-LacZ*)	—	56	134	2.4	R
C.							
W109-9C X Y349	(*hpt1/HPT1*)	P115 (*ADE1-LacZ*)	—	2	25	12.5	S
W109-9C X Y531	(*hpt1/bra6-2*)	P115 (*ADE1-LacZ*)	—	21	32	1.5	R

[a] RF, repression factor.
[b] 8AG, 8-azaguanine.
[c] R and S, resistant and sensitive, respectively.

establishing that the total flux between the two routes is critical for the repression mechanism.

To exert its regulatory effect, adenine has to be converted into ADP but not into dADP: Results presented in the previous sections strongly suggest that adenine once inside the cell needs to be metabolized into AMP to exert its regulatory role. We have tested whether transformation of AMP into ADP was required for adenine repression. For this purpose, we have used a strain disrupted at the *ADK1* locus (KONRAD 1992). In this strain only 10% of wild-type AMP kinase activity can be detected in a crude extract (KONRAD 1992). Results presented in Table 8 clearly show that expression of an *ADE1-LacZ* fusion in this mutant strain is totally unaffected by addition of adenine in the medium. Therefore we conclude that AMP has to be converted into ADP for correct transduction of the repression signal. Finally, we have tested whether reduction of ADP into dADP was required for repression by adenine. This was done by two different approaches. First, we used a temperature-sensitive allele (named *crt6-68*) of the *RNR2* gene, which encodes a subunit of ribonucleotide reductase (ZOU and ELLEDGE 1992). This strain was cured for the pZZ13 plasmid and then cotransformed with an *ADE2*-CEN vector (pASZ11, STOTZ and LINDER 1990) to make it Ade^+ and with the P473 plasmid carrying the *ADE1-LacZ* fusion. Expression of *ADE1-LacZ* in this mutant strain and in the wild-type isogenic strain was then measured after growth at 30°. This temperature was chosen because at 30° the Y221 mutant strain grows much slower than the isogenic wild-type strain (named Y203), indicating that under these conditions synthesis of dNTPs is most probably limiting for growth. Results presented in Table 8 show that the *crt6-68* mutation has no effect on repression by adenine. The same result was obtained when ribonucleotide reductase activity was blocked using hydroxy urea (HU). A strain carrying an *ADE1-LacZ* fusion integrated at the *ADE1* locus was grown in SD medium with or without adenine that contained increasing concentrations of HU (5–80 mM). After 13 h expression of the fusion was monitored and no effect of HU on regulation of the fusion could be detected even under conditions where growth is severely affected by the drug (data not shown).

DISCUSSION

To investigate the signalling pathway controlling adenine responsive genes, we have isolated constitutive mutants that relieve the transcriptional repression of *ADE* genes by adenine. A full understanding of the pathway will require the identification of the following: (1) the signal (the effector molecule) (2) the transcription factors responding to the signal and (3) the protein factors that are required for the perception of the signal and for its transduction to the transcription factors. Our phenotypical and molecular analysis of the *bra* mutants sheds light on the two first points.

ADP or a derivative of ADP is the effector molecule: Because *S. cerevisiae* does not take up external nucleotides, the nature of the effector cannot be simply tested by adding nucleotides to the medium. Our genetic analysis provides strong clues about the identity of the effector molecule. First, the fact that *BRA7* is allelic to *FCY2*, the gene encoding purine permease, indicates that purines need to be taken up into cells to trigger repression of biosynthetic genes. It is unlikely that purine bases themselves are the effector molecules because mutations that block their metabolism abolish their regulatory effect (for example, *hpt1* mutation in the case of hypoxanthine or a double *aah1 apt1* mutation in the case of adenine). Second, our results suggest

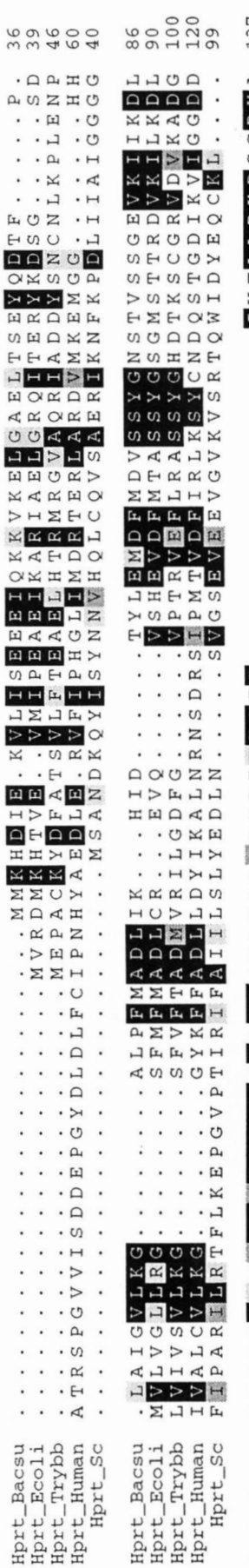

FIGURE 6.—Comparison of hypoxanthine phosphoribosyl transferase sequences from various organisms. The amino acid sequence deduced from the nucleotide sequence of the *S. cerevisiae HPT1* gene (Hprt_Sc) was compared with HPRT sequences from *B. subtilis* (Bacsu), *E. coli* (Ecoli), trypanosoma (Trybb) and human. Identies and similarities are highlighted in black and gray boxes, respectively. Periods indicate the gaps created for alignment.

that the major route for adenine utilization under the tested conditions is its deamination into hypoxanthine followed by transformation into IMP. This result is in good agreement with direct measurement of enzymatic activities (DEELEY 1992). Third, the fact that mutations that decrease AMP synthesis (mutations at the *ADE12* and *ADE13* loci) were obtained in this screen strongly suggests that AMP plays an important role in the adenine repression process (see Figure 2). This conclusion is strengthened by the experiment in which overexpression of *APT1* in the *hpt1* mutant restores wild-type repression. Finally, mutation of the gene encoding AMP kinase, *ADK1,* abolishes transcriptional regulation by adenine. This result suggests that ADP or a derivative of ADP is the effector.

The basis for the different levels of constitutive expression of the *ADE* genes in the *bra* mutants is not yet clear. Mutations in the same complementation group usually fall into the same phenotypic class (Table 3). There are several plausible explanations for the partial derepression observed in some of the *bra* mutants. We know from measuring adenylosuccinate lyase activity that *bra1-2* and *bra1-3* are partial loss of function alleles (Table 4). The residual enzymatic activity probably accounts for the partial derepression. This is clearly not the case for *bra6,* because the *HPT1* null allele leads to a partial derepression phenotype (Table 5B). We believe that the most likely explanation for the *bra6* partial phenotype is that some AMP can be synthesized via the APRT route. If *bra1* and *bra6* mutants were simply affecting the same process (accumulation of AMP), they would be expected to belong to the same phenotypic class. However, this is not the case. The *bra6-2* mutant belongs to the first class along with mutants that are blocked in adenine uptake (*bra7* mutants, Table 3) or utilization (*aah1 apt1* mutants, Table 6B). By contrast, *bra1* mutants belong to class 3, those mutants where expression of *ADE* genes is increased relative to wild type even under derepression conditions. This discrepancy can be explained by the fact that adenylosuccinate lyase, the product of the *BRA1* gene, participates both in the *de novo* synthesis of purines and in purine salvage. Mutation of this locus is therefore expected to produce a more severe starvation for AMP than a mutation that only affects the salvage pathway. Characterization of other complementation groups should help explain the phenotypic differences between the mutants.

One surprising result obtained here is the ability of guanine to cause transcriptional repression of adenine biosynthetic genes. Guanine is not able to support the growth of mutants deficient in *de novo* purine biosynthesis. It was therefore thought that *S. cerevisiae* lacks GMP reductase activity (see discussion in BURRIDGE *et al.* 1977). Our results indicate that guanine exerts a partial repression effect that requires HGPRT activity (see Table 6A). Since this repression by guanine is abolished

TABLE 6

Expression of the *ADE1-LacZ* fusion in the presence of different purine bases in strains carrying different combinations of *hpt*, *aah1* and *apt1* mutations

Strain	Relevant genotype	βGal activity 0	ade	gua	hyp
A.					
PLY122	Wild type	98	16	40	16
Y508	*hpt1*	149	121	126	133
Y511	*apt1*	145	24	54	22
Y520	*aah1*	152	23	81	29
B.					
PLY122	Wild type	68	18	55	32
Y550	*apt1 aah1*	90	92	53	24
Y551	*apt1 aah1*	100	104	54	26
Y552	*apt1 aah1*	83	81	43	19

0, growth on SD medium containing no purine base; ade, gua and hyp, growth on SD medium supplemented with adenine, guanine or hypoxanthine, respectively.

in a *ade13* mutant (data not shown), this suggests the existence of a GMP reductase activity providing a sufficient amount of IMP and AMP to cause repression (see Figure 2). This weak activity might not be sufficient to allow *ade* mutants to grow in the presence of guanine as a purine source. The existence of such an enzymatic activity is supported by studies on intracellular purine content of cells fed with radioactive guanine (BURRIDGE *et al.* 1977).

What are the protein factors involved in the signal transduction pathway? We have shown that an *ADE2-lacZ* fusion mutated for its Bas1p binding sites is not regulated by adenine and is not derepressed by the *bra* mutations. This suggests an important role for Bas1p in regulation as well as activation. Since Bas1p carries a potential nucleotide binding site in its protein sequence (TICE-BALDWIN *et al.* 1989), it is tempting to propose that the effector could bind to Bas1p, directly affecting its capacity to bind DNA, interact with other factors, or activate transcription. The central role for Bas1p in this process is confirmed by the fact that all the genes regulated by adenine isolated so far are also activated by Bas1p. If there is a direct interaction between Bas1p and the effector, the signal transduction pathway would converge on the transcription factor and it would therefore be expected that only a few dominant mutations at the *BAS1* locus could lead to the derepression phenotype. We have isolated several dominant mutations in our screen. It will be interesting to determine if some of these mutations are in *BAS1*.

TABLE 7

Effect of overexpression of the *APT1* gene on expression of the *ADE1-LacZ* fusion in the *bra6-2* mutant strain

Plasmid	βGal activity +ade	−ade	RF
YEp13 (control *LEU2*, 2μ)	39	73	1.9
pCG3 (*APT1* in YEp13 *LEU2*, 2μ)	11	66	6.0

RF, repression factor.

TABLE 8

Effect of mutations in the *ADK1* and *RNR2* genes on expression of an *ADE1-LacZ* fusion

Strain	Relevant genotype	βGal activity −ade	+ade	RF
AH215	Wild type	412	25	16.7
AH215*adk1*	*adk1::HIS3*	334	296	1.1
Y203	Wild type	29.8	2.8	10.8
Y221	*rnr2*	19.0	1.9	10.0

RF, repression factor.

Is adenylosuccinate synthetase a bifunctional protein? From previous work (DORFMAN *et al.* 1970, LOMAX and WOODS 1970) it has been proposed that the *ADE12* gene could encode both catalytic and regulatory functions. This conclusion was based on the isolation of prototrophic regulatory mutants of adenylosuccinate synthetase. This conclusion is at variance with our results. Although we have isolated a mutant (*bra9-1*) that has lost both adenylosuccinate synthetase activity and regulatory properties, we have also found that mutations at the *ADE13* locus are similarly deregulated. Deregulation is therefore not specific to *ADE12*, but is observed with any block in the pathway from IMP to AMP. Furthermore, we have shown, for some alleles of *ADE13*, that decreased enzymatic activity leads to a derepressed phenotype but no growth requirement for adenine. It would be interesting to know whether the *ade12* prototrophic regulatory mutants previously described have wild-type levels of adenylosuccinate synthetase activity.

Yeast as a model to study purine metabolism regulation in higher eucaryotes: The genes that we have shown play central roles in yeast adenine regulation correspond to important human disease genes. The best understood example at the molecular level is Lesch-Nyhan syndrome, a syndrome whose symptoms include hyperuricemia, severe mental retardation and automutilation (LESCH and NYHAN 1964). Lesch-Nyhan syndrome results from the absence of HPRT activity due to mutations in the HPRT gene (SEEGMILLER *et al.* 1967). Patients with a partial defect in HPRT activity have also been described, and they develop hyperuricemia but not the other features of Lesch-Nyhan syndrome (KELLEY *et al.* 1967). These HPRT-deficient patients show an increased synthesis of purine nucleotides and it was proposed that this could be due to increased

PRPP levels due to the lack of salvage of hypoxanthine and guanine by HPRT (ROSENBLOOM *et al.* 1968). The excess of PRPP would be shunted into the *de novo* pathway leading to increased purine biosynthesis. Lack of adenylosuccinate synthetase and adenylosuccinate lyase have also been shown to be associated, respectively, with purine oversecretion (ULLMAN *et al.* 1982) and mental retardation (STONE *et al.* 1992).

Our findings in yeast suggest an appealing hypothesis to explain the purine overproduction in Lesch-Nyhan syndrome and related disorders. We found that mutations in the yeast genes encoding HPRT, adenylosuccinate synthetase or adenylosuccinate lyase lead to derepressed synthesis of the *de novo* pathway enzymes. Furthermore, purine secretion has been described for certain alleles of the *ade12* locus (LOMAX and WOODS 1970) and can be associated with increased *de novo* synthesis of purines (BURRIDGE *et al.* 1978). Therefore mutations at these loci could lead to purine overproduction by deregulating the synthesis of the *de novo* pathway rather than by increasing substrate (PRPP) availability. It would be interesting to test whether deregulation and overexpression of the *de novo* pathway enzymes is also observed in human cell lines deficient in either HPRT, adenylosuccinate lyase or adenylosuccinate synthetase.

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