Cyclic AMP-Dependent Protein Kinase Inhibits *ADH2* Expression in Part by Decreasing Expression of the Transcription Factor Gene *ADR1*

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In *Saccharomyces cerevisiae*, the unregulated cyclic AMP-dependent protein kinase (cAPK) activity of *bcy1* mutant cells inhibits expression of the glucose-repressible *ADH2* gene. The transcription factor Adr1p is thought to be the primary target of cAPK. Here we demonstrate that the decreased abundance of Adr1p in *bcy1* mutant cells contributes to the inhibition of *ADH2* expression. Activation of *ADH2* transcription was blocked in *bcy1* mutant cells, and UAS1, the Adr1p binding site in the *ADH2* promoter, was sufficient to mediate this effect. Concurrent with this loss of transcriptional activation was an up to 30-fold reduction in the level of Adr1p. Mutating the strong cAPK phosphorylation site at serine 230 did not suppress this effect. Analysis of *ADR1* mRNA levels and *ADR1-lacZ* expression suggested that decreased *ADR1* transcription was responsible for the reduced protein level. In contrast to the *ADH2* promoter, however, deletion analysis suggested that cAPK does not act through a discrete DNA element in the *ADR1* promoter. The amount of Adr1p found in *bcy1* mutant cells should have been sufficient to support 23% of the wild-type level of *ADH2* expression. Since no *ADH2* expression was detectable in *bcy1* mutant cells, cAPK must also act by other mechanisms. Overexpression of Adr1p only partially restored *ADH2* expression, indicating that some of these mechanisms may impinge upon events at or subsequent to the *ADR1*-dependent step in *ADH2* transcriptional activation.

The cyclic AMP (cAMP) signal transduction pathway plays an important role in triggering changes in gene expression that are responsible for long-term adaptation to fluctuations in environmental conditions. The penultimate effector of this pathway, cAMP-dependent protein kinase (cAPK), can influence levels of gene expression by either positively or negatively controlling transcription. While its role in the positive control of transcription has been intensively studied, much less is known about its role in negative control. In the yeast *Saccharomyces cerevisiae*, activation of cAPK inhibits expression of genes involved in the response to physical stresses and nutrient limitation, such as *SSA3*, *UBI4*, *ADH2*, and *CTT1* (5, 9, 12, 41) and others involved in cell cycle control and differentiation, such as *IME1* and the G1 cyclins *CLN1* and *CLN2* (3, 32). We have been studying the inhibition of *ADH2* expression to gain insights into the molecular mechanisms involved in the negative control of transcription by cAPK.

Maximal expression of the *ADH2* gene, which encodes the glucose-repressible isozyme of alcohol dehydrogenase, ADHII, requires the transcription factor Adr1p (13, 18). Two Adr1p monomers bind noncooperatively via Cys₂His₂ class zinc finger domains to the palindromic sequence of UAS1 in the *ADH2* promoter (45). When yeast cells are starved for glucose, this Adr1p-UAS1 complex activates *ADH2* transcription, which ultimately results in an over 400-fold increase in ADHII activity. Cells with mutations that increase the level of cAPK activity are unable to accumulate the maximal level of ADHII activity (12, 17, 19).

The cAPK holoenzyme consists of a regulatory subunit homodimer and two inactive catalytic subunit monomers (42). Binding of cAMP to the regulatory subunits releases active

catalytic monomers capable of phosphorylating specific protein substrates. In *S. cerevisiae*, the *BCY1* gene encodes the regulatory subunit of cAPK and there *TPK* genes encode isozymes of the catalytic subunit (46). *ADH2* expression is inhibited in cells having a deletion of the *BCY1* gene, and this inhibition can be ameliorated by mutations in the *TPK* genes (12, 17, 19). This suggests that unregulated cAPK activity inhibits an important step required for the activation of *ADH2* expression.

Several observations suggest that Adr1p might play a key role in this important step. First, *ADR1*-dependent, but not *ADR1*-independent, *ADH2* expression is inhibited by unregulated cAPK activity. *ADH2* expression in repressed cells carrying specific *ADR1^c* alleles is inhibited while *ADR1*-independent expression in *cre1lsp10* mutant cells is not (12, 19). In addition, the activity of an *ADH2* promoter in which UAS1 has been precisely deleted is not inhibited (17). Second, expression of a different *ADR1*-dependent gene, *CTA1*, which has a functional UAS1 homolog in its promoter, is also inhibited by unregulated cAPK activity (11, 39). Adr1p expressed from the strong *ADH1* promoter in *bcy1* mutant cells has the same apparent binding affinity for UAS1 as does Adr1p expressed in wild-type cells (43). Therefore, the defect in *ADR1*-dependent activation is probably not caused by the inability of Adr1p to bind UAS1 due to phosphorylation by cAPK.

The mechanism by which cAPK inhibits *ADR1*-dependent transcription has yet to be firmly established. One extensively investigated possibility is that cAPK phosphorylates Adr1p and thereby inactivates it. The amino acid sequence of Adr1p has potential cAPK phosphorylation sites located at serines 170, 180, 212, and 230 (25). The high-affinity phosphorylation site at serine 230 could be phosphorylated in vitro by the catalytic subunits of both yeast and mammalian cAPK, and Adr1p was found to be phosphorylated in vivo (12, 20, 43). Mutations at or near serine 230 allowed Adr1p to activate *ADH2* expression in repressed cells where Adr1p is thought to be inactive (12). Several of these *ADR1^c* mutations decreased or eliminated

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TABLE 1. Yeast strains used in this study

Strain ^a	Genotype	Source
CMY215	<i>MATa ade2 can1 his3 lys2 trp1 ura3</i>	Carl Mann
CMY215Δ1	<i>MATa ade2 adr1 Δ2 can1 his3 lys2 trp1 ura3</i>	This study
CMY466	<i>MATa ade2 bcy1::LEU2 his3 leu2 lys2 tpk1::URA3 tpk2::HIS3 tpk3^{ts} trp1 ura3</i>	Carl Mann
KDY41 ^b	<i>MATa ade2 bcy1::URA3 can1 his3 lys2 trp1 ura3</i>	This study
KDY42	<i>MATa ade2 can1 his3 lys2 snf1::URA3 trp1 ura3</i>	This study
KDY46	<i>MATa ade2 can1 his3 lys2 trp1 URA3::Ylp16ADRI-lacZ</i>	This study
KDY47 ^c	<i>MATa ade2 bcy1::HIS3 can1 his3 lys2 trp1 URA3::Ylp16ADRI-lacZ</i>	This study
KDY73	<i>MATa ade2 can1 his3 lys2 mig1-82::URA3 trp1 ura3</i>	This study
KDY80	<i>MATa ade2 ADH2::Ylp23ADH-lacZ can1 his3 leu2 lys2 trp1 ura3</i>	This study
KDY90 ^b	<i>MATa ade2 ADH2::Ylp23ADH-lacZ bcy1::URA3 can1 his3 leu2 lys2 trp1 ura3</i>	This study
KDY94 ^c	<i>MATa ade2 adr1Δ2 bcy1::HIS3 can1 his3 lys2 trp1 ura3</i>	This study

^a All strains are either isogenic or congenic to CMY215.

^b *bcy1::URA3* is equivalent to *sra1-20*.

^c *bcy1::HIS3* is equivalent to *sra1-21*.

phosphorylation in vitro and made *ADH2* expression more resistant to inhibition by unregulated cAPK. These observations were interpreted as evidence for the inactivation of Adr1p by cAPK-mediated phosphorylation of serine 230.

However, subsequent observations have suggested that phosphorylation of serine 230 is insufficient to completely account for inhibition by cAPK. Several other *ADRI*^c mutations did not appear to inhibit phosphorylation by cAPK. Model synthetic peptide substrates containing these mutations were phosphorylated with wild-type kinetics by yeast cAPK (19). Additionally, cells lacking measurable cAPK activity did not express *ADH2* under repressing growth conditions where cAPK is presumed to be activated (19, 20). Therefore, the *ADRI*^c phenotype does not appear to be equivalent to the lack of phosphorylation by cAPK. Although these observations do not conclusively rule out the possibility that phosphorylation at serine 230 contributes to the inactivation of Adr1p, they do suggest that cAPK may inhibit Adr1p-dependent gene expression by other mechanisms. In this paper, we describe the first direct evidence of an alternate mechanism. We demonstrate that activated cAPK inhibits *ADRI* transcription, causing the level of Adr1p to fall below that required for wild-type levels of *ADH2* expression.

MATERIALS AND METHODS

Yeast strains and culture conditions. The strains of *S. cerevisiae* used in this study are listed in Table 1. All strains are isogenic with CMY215 except for CMY466, KDY80, and KDY90, which are congenic. Yeast cells were transformed by a modified version of the lithium acetate procedure as described by Gietz et al. (22). Because we were unable to obtain transformants of strains containing a *bcy1* disruption, *bcy1* mutants containing integrated or episomal plasmids were constructed by introducing the *bcy1* allele into cells already containing the plasmid of interest. Disruption alleles were introduced into cells by the one-step gene disruption technique as described by Rothstein (37). The *sra1-20::URA3* and *sra1-21::HIS3* alleles were introduced into cells by transforming with *Bam*HI digests of plasmids p759 or pBR322-*bcy1::HIS3*, respectively (10). The *snf1::URA3* allele was similarly introduced by transformation with an *Eco*RI digest of plasmid pST70 (44). Both *BCY1*- and *SNF1*-disrupting plasmids were kindly provided by Kelly Tatchell. Integration of these alleles at the appropriate chromosomal location was confirmed by Southern blot analyses (2). Cells with *bcy1* disruption alleles were also monitored phenotypically by testing for impaired growth on yeast extract-peptone (YEP) agar containing 2% glycerol (YEPG), heat shock sensitivity, and flocculation after prolonged incubation in YEPD broth (see below). Cells with the *snf1* disruption were monitored for the inability to grow on YEPG agar (see below) and the inability to accumulate ADHII protein after 24 h in YEPE broth. The *mig1-82::URA3* allele was introduced into cells by transforming with a *Sac*I digest of pJN41 (34). This plasmid was kindly provided by Hans Ronne. Integration of the *mig1-82* allele at the appropriate chromosomal location was confirmed by PCR with the appropriate diagnostic primers as described by Ling et al. (28). The primers used to analyze the pJN41 transformants were MIG1-A (5' GGTGAGGATGAAGAAGACGG 3'), MIG1-B (5' TTTGATTTATCTGCACCGCC 3'), and LP-2 (5' GAAGAT GCGGCCAGCAAAAC 3'). The level of invertase activity in cells with the

mig1-82 allele (KDY73) was not fully repressed in glucose medium, being about 1/10 of the level found in fully derepressed cells. This is consistent with previously reported values for invertase activity in *mig1* mutant cells having the same S288C strain background as used in our experiments (48). The *adr1Δ2* allele was introduced into CMY215 as described previously (21). The presence of this deletion was physically confirmed by Southern blot analysis of genomic DNA and phenotypically monitored by using the inability to accumulate ADHII protein after 24 h in YEPE broth. The integrating reporter plasmid Ylp23ADH2-*lacZ* has *ADH2* sequences from the upstream *Bam*HI site to the first *Eco*RV site in the *ADH2* open reading frame fused to the *Escherichia coli lacZ* open reading frame (7). It was targeted to the *ADH2* locus by digestion with *Sph*I. Ylp16ADRI-*lacZ*, described previously (7), was targeted to the *URA3* locus by digestion with *Sma*I (21). Transformants containing a single integrated copy of each reporter were identified by Southern blot analyses.

Media and culture conditions were as previously described (50). Yeast cells were grown in YEP medium or, when appropriate, synthetic (S) medium lacking amino acids and containing 0.4% casamino acids to select for *TRP1* episomal plasmids. Repressing growth medium contained 5% glucose (YEPD or SD medium), and derepressing growth medium contained 3% ethanol (YEPE medium) as carbon sources. Repressed cells were prepared by growing cultures in YEPD medium, or SD medium where appropriate, to the late exponential phase. Derepressed cells were prepared by washing the cells in a portion of each repressed culture with cold YEP broth, suspending them in YEPE broth, and then incubating them for an additional 24 h. Under these conditions, ADHII protein levels are maximally derepressed in wild-type cells. When other strains were compared to CMY466, which is temperature sensitive for growth at 37°C and somewhat impaired for growth at 30°C, all of the strains in that experiment were grown at 23°C.

Plasmid constructions. The procedures for manipulating DNA were performed essentially as described by Ausubel et al. (2). *CEN* plasmids containing the complete *ADRI* promoter and open reading frame were prepared by inserting a 6.1-kb *Bam*HI-*Bst*EII fragment containing *ADRI* sequences and a *Sal*I linker at the *Bst*EII end between the *Bam*HI and *Sal*I sites of pBC3T1 (4) in several steps. First, a 5-kb *Bam*HI fragment containing 1.8 kb of 5' untranslated flanking sequence and 80% of the *ADRI* open reading frame was ligated into the *Bam*HI site of pBC3T1. Then the *ADRI* sequence of one ligation product, which contained an insert oriented with the upstream sequence of *ADRI* adjacent to *CEN3*, was extended to include the 3' end of the gene. To accomplish this, an *Sph*I-*Sal*I vector fragment was ligated to a 0.54-kb *Sph*I-*Nde*I fragment which spans the region of the *ADRI* open reading frame containing potential cAPK phosphorylation sites and a 3.4-kb *Nde*I-*Sal*I fragment which contains sequences up to the *Bst*EII site 3' of the open reading frame. One product of this three-way ligation was plasmid pKD16. The *ADRI*-5^c mutation was introduced into the open reading frame by substituting an *Sph*I-*Nde*I fragment containing the R228K mutation for the wild-type fragment in the three-way ligation to create plasmid pKD27.

The *ADRI-lacZ* reporter plasmid, pKD70, which has 1.8 kb of 5'-flanking sequence fused at codon 16 of the *ADRI* open reading frame to the *E. coli lacZ* gene, was prepared essentially by transferring a 5.3-kb *Kpn*I-*Sal*I fragment from Ylp16ADRI-*lacZ* to pRS314 (38). Reporter plasmids containing deletions within the *ADRI* promoter were derived from pKD70 as follows: pKD80 (Δ -555, -207), a 1.08-kb *Kpn*I-*Ssp*I fragment of *ADRI* upstream sequence was cloned into *Kpn*I-*Apa*I-digested pKD70, where the *Apa*I end of the fragment was blunted with T4 polymerase; pKD81 (Δ -453, -207), a 1.18-kb *Kpn*I-*Nar*I fragment, where the *Nar*I end was blunted with Klenow enzyme, was cloned into *Kpn*I-*Apa*I-digested pKD70, where the *Apa*I end of the fragment was again blunted; pKD78 (Δ -256, -207), pKD70 was partially digested with *Nco*I, completely digested with *Apa*I, treated with T4 polymerase, and then self-ligated; pKD79 (Δ -1201, -261), a 0.5-kb *Kpn*I-*Apa*I fragment from pKD67 (described

below) in which a 0.9-kb *NcoI* fragment had been deleted was cloned into *KpnI*-*ApaI*-digested pKD70; pKD75 (Δ -455, -314), a 1.2-kb *KpnI*-*ApaI* fragment from pKD67 in which a 0.14-kb *NarI* fragment had been deleted was cloned into *KpnI*-*ApaI*-digested pKD70; pKD76 (Δ -455, -343), a 1.3-kb *KpnI*-*ApaI* fragment from pKD67 in which a 0.11-kb *NarI* fragment had been deleted was cloned into *KpnI*-*ApaI*-digested pKD70. Plasmid pKD67 consists of a 3-kb *ClaI* fragment containing the *ADR1* upstream sequence from YIp16*ADR1-lacZ* cloned into the polylinker of pGEM7-Zf(+) (Promega, Madison, Wis.).

CEN plasmids carrying *ADR1* genes with promoter deletions were constructed from the *ADR1-lacZ* reporter plasmids described above. Plasmids pKD84, pKD85 (Δ -1201, -261), and pKD86 (Δ -455, -343) were made by cloning a 5.1-kb *ApaI*-*Sall* fragment containing the wild-type *ADR1* open reading frame and 3'-flanking sequence up to a *BstEII* site into the *ApaI*-*Sall* vector fragments from pKD70, pKD79, and pKD76, respectively. Plasmid pKD87 (Δ -555, -207) was created by cloning the same 5.1-kb *ApaI* (blunted)-*Sall* fragment into the *SspI*-*Sall* vector fragment of pKD70.

ADR1 expression plasmids with the *ADHI* or *CUP1* promoters were derived from a version of pYcDE2 (43) in which a 1.5-kb *SphI*-*PvuII* had been deleted. A 10-bp *Sall* linker was inserted at the unique *EcoRI* site between the *ADHI* promoter and *CYC1* sequences to create the *ADHI* expression vector pKD9. Next, a *Sall* fragment beginning at the first *NciI* site upstream of the *ADR1* translation start site and ending at the *ScaI* site immediately following the end of the open reading frame was cloned in several steps into the *Sall* site to create pKD17. In this plasmid, the *ADR1* gene is oriented in the same direction as the *ADHI* promoter. Next, the 3' *Sall* site was selectively destroyed by blunting with Klenow enzyme. Then, a 1.8-kb *XhoI*-*SacI* fragment containing the 5' end of the *ADR1* coding sequence and a *BamHI*-*Sall* linker inserted in frame after codon 16 was cloned into *Sall*-*SacI*-digested vector to create plasmid pKD43. The *XhoI* end of the fragment was constructed by blunting the *NciI* end and ligating on *XhoI* linkers. The *CUP1* promoter expression plasmid, pKD44, was constructed similarly. First, a 5.6-kb *PvuII*-*EcoRI* vector fragment from pYcDE2 was ligated to a 0.4-kb *HincII*-*EcoRI* fragment containing the *CUP1* promoter from plasmid pUC:CRB, which was kindly provided by Dennis Thiele. *ADR1* coding sequence including the *BamHI*-*Sall* linker at codon 16 was then cloned into the *Sall* site between the *CUP1* promoter and *CYC1* sequences as described above.

ADH native-gel analyses and β -galactosidase enzyme assays. Native whole-cell extracts were prepared by vortexing cells in ADH lysis buffer (30 mM Tris [pH 7.5], 85 mM KCl, 3 mM magnesium acetate, 25% glycerol) with glass beads. The protein concentration in each extract was determined by the method of Lowry et al. (30). Electrophoresis and in situ detection of isozymes by chromogenic staining for ADH activity were carried out essentially as described by Williamson et al. (49). Activity-stained gels were dried between two sheets of cellophane, and then digital images were prepared with either the UVP ImageStore 5000 system (UVP, San Gabriel, Calif.) or a Microtek Scanmaker flatbed scanner. Digital images were quantitatively analyzed with the Gel Plotting Macros of the NIH Image program (version 1.59) essentially as described in the Gel Plotting Macros documentation (DCRT-NIH, Bethesda, Md.).

Assays of β -galactosidase activity were performed on permeabilized whole cells as described by Guarente (24).

Western blot analyses of Adr1p and general transcription factors. Denatured whole-cell extracts were prepared as previously described (21). Nitrocellulose protein blots were sequentially probed with polyclonal antibodies raised against amino acids 335 to 740 of Adr1p, TATA-binding protein (TBP), Toa1p, and Sua7p. The anti-TBP, -Toa1p, and -Sua7p sera were kindly provided by members of S. Hahn's lab. After each probing, antibodies were removed from the blots by washing with stripping buffer (100 mM β -mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris [pH 7.5]) at 50°C for 1 h. Adr1p bands were visualized by chemiluminescence with ECL reagents supplied by Amersham (Arlington Heights, Ill.). Digital images of exposed X-ray films were prepared and analyzed as described above for ADH native gels.

RNA preparation and analysis. Total yeast RNA was prepared as described by Cross and Tinklenberg (15) and separated on a 1% agarose-formaldehyde gel. RNA was blotted to Zeta-Probe membrane (Bio-Rad, Hercules, Calif.) by a version of the alkaline transfer method for DNA described by Reed and Mann (36) as modified by Bio-Rad for RNA. *ADH2* and *ADR1* mRNAs were detected as described previously (21, 26).

RESULTS

Unregulated cAMP-dependent protein kinase inhibits UAS1-mediated transcription of *ADH2*. Unregulated cAPK activity can inhibit the accumulation of ADHII protein in derepressed cells. Several observations suggest that this inhibition occurs at the level of *ADH2* transcription. *ADR1^c* mutations or expression of Adr1p at high levels partially suppressed this inhibition (12, 19, 43). Also, Adr1p-independent expression of the *ADH2-11a* allele, which contains an exact deletion of UAS1 in the *ADH2* promoter, was unaffected by unregulated cAPK (17). To obtain more direct evidence that cAPK

FIG. 1. Effect of unregulated cAPK activity on *ADH2* expression. *ADH2* expression was first examined by native-gel analysis of ADH isozyme levels. Native whole-cell extract containing 100 μ g of protein was loaded in each lane of a nondenaturing polyacrylamide gel. The panel is a picture of the relevant portion of the native gel after staining for ADH activity. The slower-migrating band is the ADHI isozyme, while the faster-migrating band is the ADHII isozyme. The amount of ADHII in each lane is listed as a percentage of that found in derepressed CMY215 cells. In a separate experiment, *ADH2* promoter activity in cells containing the YIp23*ADH2-lacZ* reporter plasmid was assessed. β -Galactosidase activities were measured in permeabilized whole cells and are reported in Miller units below each lane. Data for KDY80 are the mean values of three independent assays of two congenic siblings derived from a diploid containing a single integrated copy of YIp23*ADH2-lacZ*. Data for KDY90 are the mean values for three independent *bcy1* mutants derived from KDY80. Standard deviations for these values are less than 15%. R and DR, repressed and derepressed, respectively. nd, not detectable. —, not done. Strains: *adr1*, CMY215 Δ 1; WT, CMY215; *bcy1*, KDY41; *bcy1 tpk1*, CMY466; WT *ADH2-lacZ*, KDY80; *bcy1 ADH2-lacZ*, KDY90.

acts at the level of *ADH2* transcription, expression of an *ADH2-lacZ* reporter gene and the accumulation of *ADH2* mRNA in cells with a disrupted *BCY1* gene was examined. Since these cells have a complete set of wild-type *TPK* genes, they were expected to show a stronger defect in *ADH2* expression than had been observed previously for strains with an incomplete set (12, 17, 19).

Yeast cells with the *bcy1* gene disrupted did show a much stronger defect in *ADH2* expression than had been previously reported. No ADHII protein could be detected in *bcy1* mutant cells under derepressing conditions (Fig. 1). However, ADHII protein accumulated to the wild-type level in *bcy1* mutant cells with mutations in all three *TPK* genes, confirming that cAPK activity is required for inhibition. The level of *ADH2* mRNA and expression of an *ADH2-lacZ* reporter gene in *bcy1* mutant cells indicated that this failure to accumulate ADHII protein occurred because *ADH2* transcription was severely inhibited. *ADH2* mRNA was not detectable in *bcy1* mutant cells even after 24 h in derepressing medium (data not shown). In wild-type cells, however, *ADH2* mRNA could be detected as soon as 1 h after initiating derepression and reached a maximum level by 12 h. Expression of an *ADH2-lacZ* reporter reflected this defect in *ADH2* mRNA accumulation (Fig. 1). β -Galactosidase activity increased over 100-fold when wild-type cells with the reporter were derepressed. However, less than a twofold increase in expression was observed for *bcy1* mutant cells. This shows that sequences upstream of codon 23 in the *ADH2* open reading frame are sufficient to confer inhibition by unregulated cAPK.

Of these upstream sequences the binding site for Adr1p, UAS1 appears to be the primary mediator of inhibition. Expression of a *CYC1-lacZ* reporter gene in *bcy1* mutant cells indicated that UAS1 was sufficient to confer inhibition by cAPK upon a heterologous promoter. β -Galactosidase expression from the reporter plasmid p2DY-T, in which the native *CYC1* UASs have been replaced with two tandem UAS1 elements, is normally high in derepressed cells (21, 50). For the yeast strain used in this study, β -galactosidase expression was 800-fold higher than in repressed cells (Table 2). However, in

TABLE 2. Effect of unregulated cAPK activity on UAS1-mediated expression

Plasmid	UAS elements	β -Galactosidase activity ^a (Miller units)			
		Repressed		Derepressed	
		<i>BCY1</i>	<i>bcy1</i>	<i>BCY1</i>	<i>bcy1</i>
p2DY-T	2× UAS1	0.8 ± 0.2	0.6 ± 0.8	810 ± 30	1.4 ± 0.1
pM2-T	2× UAS2	4.5 ± 0.7	3.3 ± 0.5	4.9 ± 0.4	3.7 ± 0.2
pLG669Z	<i>CYC1</i>	390 ± 20	85 ± 7	1,330 ± 40	84 ± 5
pLG-S/X	None	0.3 ± 0.1	<0.2	0.4 ± 0.1	0.9 ± 0.1

^a Values (± standard deviation) are the means for at least three independent transformants.

bcy1 mutant cells expression was only 2-fold higher. This suggests that UAS1 is the main target of cAPK in the reporter plasmid. To rule out the possibility that other plasmid sequences were mediating the inhibitory effect of cAPK, expression of the same reporter gene containing UAS2 elements instead of UAS1 was examined. A comparison of pM2-T and the reporter lacking UAS elements, pLG-S/X, showed that UAS2 sequences activated β -galactosidase expression 5- to 10-fold. However, β -galactosidase expression was decreased less than twofold in *bcy1* mutant cells under both repressing and derepressing growth conditions. Therefore, it is unlikely that cAPK acts through other sequences in the reporter plasmid. It is also unlikely that the primary effect of cAPK is to activate a pathway that promotes the degradation of β -galactosidase protein, since relatively high levels of β -galactosidase were observed in *bcy1* mutant cells containing the pLG669-Z reporter plasmid. Even though these levels were significantly lower than those in wild-type cells, they were still at least 60-fold higher than in *bcy1* mutant cells containing the UAS1 reporter plasmid.

Unregulated cAPK activity significantly decreases the level of Adr1p. Since UAS1 appeared to be the primary element in the *ADH2* promoter mediating the inhibition by unregulated cAPK activity and Adr1p is required for maximal activation of UAS1-mediated expression (50), the effect of unregulated cAPK activity on the level of Adr1p was examined. Western blot analysis of denatured whole-cell extracts from repressed and derepressed cells showed that *bcy1* mutant cells have substantially less Adr1p than wild-type cells do (Fig. 2A). Adr1p was 30-fold less abundant in *bcy1* mutant cells than in wild-type cells under derepressing conditions. Although Adr1p was easily detectable in repressed wild-type cells, only a very low level was observed in *bcy1* mutant cells. This level of protein was below the threshold required for accurate measurement by our imaging equipment. Therefore, the amount of Adr1p in repressed *bcy1* mutant cells must be at least eightfold lower than in wild-type cells. As was seen for the inhibition of *ADH2* expression (Fig. 1A), cAPK activity was also required for this effect (Fig. 2B). Although in this particular experiment no Adr1p was detectable in extracts from *bcy1* mutant cells, a substantial amount of Adr1p was found in *bcy1 tpk* mutant cells. The amount of Adr1p was restored to an approximately wild-type level in derepressed cells and to a higher-than-wild-type level in repressed cells. This higher-than-wild-type level probably does not reflect enhanced *ADRI* transcription, because expression of an *ADRI-lacZ* reporter gene was identical to that in wild-type cells.

Adr1p is a phosphoprotein *in vivo* (19, 20) and can be phosphorylated by cAPK *in vitro* at serine 230 with high affinity (12, 20). Therefore, one way that cAPK might affect the level of Adr1p is by phosphorylating it at serine 230 and targeting it for

FIG. 2. Effect of unregulated cAPK activity on *ADRI* expression. (A) Western blot analysis of Adr1p in *bcy1* mutant cells. Proteins in denatured whole-cell extracts were separated on 12% acrylamide-SDS gels, and Western blots were prepared. The arrows indicate the position of Adr1p. The taller triangles indicate that the amount of protein loaded was increased in twofold increments from 25 to 200 μ g. The shorter triangles indicate a range of protein amounts from 12.5 to 100 μ g. Below each set of lanes is listed the relative amount of Adr1p as a percentage of the amount found in CMY215. (B) *ADRI* expression in *bcy1 tpk1 tpk2 tpk3ts* (*bcy1 tpk*) mutant cells. Western blots were prepared from 5.5% acrylamide-SDS protein gels. The taller triangles indicate that the amount of protein loaded was 12.5 to 100 μ g, while the shorter triangles indicate a range of protein amounts from 6.25 to 50 μ g. The rectangles indicate 50 μ g of protein loaded. In a separate experiment, *ADRI* promoter activity was determined with cells containing the centromeric *ADRI-lacZ* reporter plasmid pKD70. β -Galactosidase activities are reported as a percentage of the value for repressed CMY215 transformants, which was 20 Miller units. Data for CMY215 and CMY466 are mean values for three independent transformants. The datum for KDY41 was taken from Fig. 5. Standard deviations for these values are less than 20%. nd, not detectable. —, not done. Strains: WT, CMY215; *adr1*, CMY215 Δ 1; *bcy1*, KDY41; *bcy1 tpk1 tpk2 tpk3ts*, CMY466; WT *ADRI-lacZ*, KDY46; *bcy1 ADRI-lacZ*, KDY47.

degradation. To test this possibility, we examined whether the level of Adr1-5^c protein, a mutant Adr1p that is poorly phosphorylatable at serine 230, was significantly lower in *bcy1* mutant cells. It was shown previously that even though this protein is much less abundant than the wild-type protein, it can activate *ADH2* expression in repressed cells (21). The Western blot in Fig. 3A confirms this observation and shows that the levels of both Adr1p and Adr1-5^c protein were reduced by a similar four- to fivefold in repressed *bcy1* mutant cells. Along with this reduction occurred a concomitant loss of constitutive *ADH2* expression (Fig. 3B). Therefore, the *ADRI*^c mutation and, presumably, reduced phosphorylation at serine 230 suppress neither the decrease in protein level nor the loss of *ADH2* expression. This suggests that cAPK may be acting at

FIG. 3. Effect of unregulated cAPK activity on the level of Adr1-5^c protein and constitutive *ADH2* expression. Denatured and native whole-cell extracts were prepared in parallel from repressed cultures of CMY215Δ1 and KDY94 containing one of the following *CEN* plasmids: pKD16, which carries the wild-type *ADR1* gene; pKD27, which carries the *ADR1-5^c* allele; or pKD8, the vector alone (—). (A) Western blot analysis of Adr1p levels. Denatured whole-cell extract containing 100 μg of protein was loaded in each lane of a 5.5% acrylamide-SDS gel. The left panel shows a 6-min exposure of the Western blot prepared from the SDS-containing gel. Below the panel are listed the relative amounts of Adr1p as a percentage of the amount found in CMY215Δ1 with pKD16. The right panel shows a 2-h exposure of selected lanes from the left panel. Below the panel are listed the relative amounts of Adr1-5^c protein as a percentage of the amount found in CMY215Δ1 with pKD27. These have been corrected for the significant *ADR1*-independent background observed after the 2-h exposure. (B) Native-gel analysis of ADH isozymes. ADH isozymes in native whole-cell extracts were analyzed as described in Fig. 1A. nd, not detectable. Strains: WT, CMY215Δ1; *bcy1*; KDY94.

least in part by a mechanism that does not involve the direct phosphorylation of Adr1p.

Yeast cells without a functional cAPK regulatory subunit do not grow in derepressing medium (10). However, two observations suggest that nonspecific effects due to growth arrest are not a significant factor in reducing the level of Adr1p. First, a deletion of the *SNF1* gene, which also prevents cells from growing in derepressing medium, does not result in a substantially lower level of Adr1p, twofold at most (data not shown). Second, the levels of numerous other proteins are not significantly lower in *bcy1* mutant cells. Ponceau S staining of the Western blots shown in Fig. 2A indicated that many abundant proteins are present at similar levels in mutant and wild-type cells (data not shown). In addition, the amounts of several proteins involved in the initiation of transcription were not substantially lower. Reprobing the Western blots of Fig. 2A for Toa1p, a subunit of TFI_{II}A, Sua7p, TFI_{II}B, and TBP, the TATA-binding subunit of TFI_{II}D, showed that repressed cells had similar amounts of each protein while derepressed cells had only a twofold-lower amount of Sua7p and an approximately equal amount of TBP (Fig. 4). Also, other experiments in our laboratory have indicated that derepression of *SUC2* expression is not significantly affected in *bcy1* mutant cells (20). Therefore, unregulated cAPK activity does not appear to interfere with general processes involved in the expression of all regulated genes.

Transcription of the *ADR1* gene is inhibited by unregulated cAPK activity. *ADR1* mRNA levels and expression of *ADR1-lacZ* reporter genes indicated that inhibition of *ADR1* transcription was primarily responsible for the decreased abundance of Adr1p. *ADR1* mRNA was undetectable in both repressed and derepressed cells having either the *sra1-20* (*bcy1::URA3*) or *sra1-21* (*bcy1::HIS3*) mutation (data not

FIG. 4. Effect of unregulated cAPK activity on the level of several general transcription factors. The Western blots shown in Fig. 2A were stripped and then reprobed consecutively with antisera raised against Sua7p, Toa1p, and TBP. Shown are the lanes in which 100 μg of total protein was loaded. Located below each lane is the relative amount of each protein listed as a percentage of the level found in CMY215. Values are not listed for Toa1p in derepressed cells because the presence of a protein that cross-reacted with the Toa1p antiserum made it impossible to accurately quantitate its level in extracts of CMY215 cells. —, not done. Strains: WT, CMY215; *bcy1*, KDY41; *adr1*, CMY215Δ1.

shown). Adr1p, however, was detectable in derepressed *bcy1* mutant cells (Fig. 2A), suggesting that *ADR1* mRNA was present but at a level below the threshold of detection for the Northern blot analysis. The use of *ADR1-lacZ* reporter genes provided a more sensitive and quantitative measurement of *ADR1* transcription. These reporter genes consisted of *ADR1* 5'-flanking sequence and the first 16 codons of the open reading frame fused to *lacZ* (7). Expression of one such reporter gene that has 1.2 kb of 5'-flanking sequence including the complete *ADR1* promoter was detectable in both repressed and derepressed *bcy1* mutant cells (Fig. 5, None). The levels of expression, however, were 9- and 25-fold lower than the levels observed for repressed and derepressed wild-type cells, respectively. These differences in reporter gene expression directly reflected the differences in Adr1p levels observed by Western blotting (Fig. 2A). Similar results were obtained with an integrated version of the reporter gene, although the β-galactosidase activities observed were three- to fourfold lower (data not shown). This shows that sequences 5' to codon 16 in the *ADR1* open reading frame can confer cAPK-inhibitable expression upon a heterologous gene. Deletion of bp -555 to -207 from the *ADR1* promoter eliminated most of the inhibition. Expression of the reporter gene containing this deletion was only 1.4- and 2.3-fold lower in repressed and derepressed *bcy1* mutant cells, respectively (Fig. 5, Δ -555, -207). This suggested that cAPK acts through sequences between bp -555 and -207 to inhibit *ADR1* transcription.

cAPK appears to act through multiple elements within the *ADR1* promoter. Analysis of additional deletions shown in Fig. 5 suggested that cAPK does not act through a discrete region of sequence within the *ADR1* promoter. In derepressed cells, expression of all reporter genes containing a portion of the sequence between bp -555 and -207 was substantially inhibited in *bcy1* mutant cells. Expression was 6- to 59-fold lower than in wild-type cells depending on the particular reporter gene being assayed. In repressed cells, expression of all reporter genes containing a portion of the sequence between bp -453 and -207 was inhibited, although the degree of inhibition was significantly lower than in derepressed cells. These observations are inconsistent with cAPK acting through a single DNA element and suggest that multiple elements within the *ADR1* promoter are being targeted.

FIG. 5. Effect of promoter deletions on the inhibition of *ADR1* expression by unregulated cAPK activity. β -Galactosidase activities were measured in permeabilized CMY215 or KDY41 cells carrying *ADR1-lacZ* reporter plasmids with deletions in the *ADR1* promoter region. Data listed here are mean values $\pm$ standard deviation for three independent transformants and are reported as a percentage of the activity observed for CMY215 carrying pKD70, which was 43 Miller units. The 5' and 3' ends of each deletion are listed in the column labeled Deletion. The stop codon of an upstream open reading frame (*U-orf*) coding for a protein of 350 amino acids with no significant homology to any other sequence in the Genbank database was located at bp -598. This open reading frame appears to be expressed because three expressed-sequence tags (ESTs) from the *S. cerevisiae* EST database developed at The Institute for Genomic Research, Gaithersburg, Md., match its 3' end. The 3'-most expressed-sequence tag begins at bp -499, suggesting that the transcriptional unit encoding the upstream open reading frame extends at least that far. —, vector without *ADR1-lacZ* sequences; ■, potential Mig1p binding sites. Strains: WT, CMY215; *bcy1*, KDY41.

cAPK appears to target at least one of these DNA elements only in derepressed cells. While expression of the Δ -555, -207 reporter gene was not greatly affected in either derepressed or repressed cells, expression of the Δ -453, -207 reporter gene was inhibited 11-fold in derepressed cells and was essentially unaffected in repressed cells. Therefore, cAPK appears to act through the 102 bp from -555 to -453 only in derepressed cells, raising the interesting possibility that cAPK inhibits the activity of a glucose-regulated element within this distal region of the promoter.

In contrast, cAPK appeared to target the sequence between bp -455 and -343 in both repressed and derepressed cells. Expression of all reporter genes missing this region was not as severely inhibited in *bcy1* mutant cells as that of the two reporter genes containing this region. In repressed cells, reporters missing this region were inhibited 1.3- to 2.6-fold while the reporters containing this region were inhibited 8- to 9-fold. In derepressed cells, the extent of inhibition ranged from 2.3- to 11-fold for reporters missing this region and 25- to 60-fold for the reporters containing this region. This region of the promoter also was particularly interesting because it has two potential Mig1p binding sites, one at bp -345 (AAAAAACC GG) and the other at bp -432 (TAAATTCGGGG). Each site has the GC box involved in forming specific protein-DNA contacts and an AT box required for high-affinity binding by Mig1p (31). When this region was deleted from the promoter, β -galactosidase expression increased 3.3-fold in repressed wild-type cells, suggesting that negative regulatory factors might be acting through it (Fig. 5, Δ -455, -314). However, expression from the intact *ADR1* promoter in repressed cells with the *mig1*- δ 2 deletion was only 1.4-fold higher than in wild-type cells (data not shown). Therefore, it is unlikely that Mig1p is solely responsible for the lower promoter activity observed in the presence of this region. Because the *mig1*- δ 2 deletion had only a weak effect on *ADR1-lacZ* expression, the ability of this deletion to suppress inhibition by cAPK in a *bcy1* mutant was not tested.

The decreased level of Adr1p in *bcy1* mutant cells contributes to but is insufficient to fully account for the lack of *ADH2* expression. By replacing the *lacZ* portion of the *ADR1* re-

porter genes with the *ADR1* open reading frame, a series of *ADR1* genes with varied levels of expression were created. These genes made it possible to examine whether the decreased level of Adr1p in *bcy1* mutant cells is sufficient to account for the lack of *ADH2* expression. If decreased abundance of Adr1p is sufficient, reducing the amount of Adr1p in wild-type cells to the level found in *bcy1* mutant cells should result in the complete loss of *ADH2* expression. The ADH native gel depicted in Fig. 6A shows that this was not the case. When the level of *ADR1* expressed in derepressed cells was eightfold lower than in wild-type cells, the level of *ADH2* expression was only slightly lower than in wild-type cells (Fig. 6A, compare Δ -1201, -261 with None). When *ADR1* expression was 80-fold lower, *ADH2* expression was still at a detectable level, 20-fold lower than in wild-type cells (compare Δ -555, -207 with None). Western blot analysis indicated that the relative level of Adr1p expressed from each promoter was consistent with the relative level of β -galactosidase activity expressed from the corresponding *lacZ* reporter plasmid (data not shown). From a plot of *ADH2* versus *ADR1* expression, it was estimated that *ADH2* expression in *bcy1* mutant cells should be 23% of the wild-type level based on the observed level of *ADR1* expression (Fig. 6B). Therefore, the level of Adr1p in *bcy1* mutant cells is unable to support the wild-type level of *ADH2* expression. This estimated level of *ADH2* expression, however, is clearly much higher than was seen for *bcy1* mutant cells (Fig. 1). Even *ADR1* expression that was 80-fold lower than the wild-type level was able to support some, albeit very low, level of *ADH2* expression in wild-type cells (Fig. 6A), whereas the 30-fold reduction in Adr1p in *bcy1* mutant cells was unable to support a detectable level of *ADH2* expression. This indicates that the low level of Adr1p in *bcy1* mutant cells is insufficient to explain the complete lack of *ADH2* expression.

Overproduction of Adr1p does not completely overcome the inhibition of *ADH2* expression caused by unregulated cAPK activity. Since decreased *ADR1* expression cannot completely account for the lack of *ADH2* expression in *bcy1* mutant cells, cAPK must be acting via at least one additional mechanism. To determine whether this action occurs prior or subsequent to

FIG. 6. Effect of decreased *ADR1* expression on *ADH2* expression. (A) Native-gel analysis of ADH isozymes. Native whole-cell extracts were prepared from CMY215 Δ 1 containing the following plasmids: pKD84, None; pKD85, Δ -1201, -261; pKD86, Δ -455, -343; pKD87, Δ -555, -207; pRS314, —. Plasmids pKD84 to pKD87 carry the complete *ADR1* open reading frame and the listed *ADR1* promoter deletions. Extracts were loaded onto a nondenaturing acrylamide gel in order of decreasing *ADR1* promoter strength based on the data in Fig. 5. The amount of ADHII in each lane of the activity-stained native gel is listed as a percentage of that found in derepressed cells containing pKD84. Below these values are listed the corresponding relative β -galactosidase activities from Fig. 5. (B) Plot of *ADR1* expression versus *ADH2* expression in derepressed cells. The relative amounts of ADHII from panel A, as a measure of *ADH2* expression, were plotted against the relative β -galactosidase activities from the *ADR1-lacZ* reporter plasmids, as a measure of *ADR1* expression. The arrow indicates the predicted level of *ADH2* expression for the level of *ADR1* expression observed in *bcy1* mutant cells. nd, not detectable.

the requirement for Adr1p, *ADH2* expression was examined in *bcy1* mutant cells expressing high levels of Adr1p. The high level of Adr1p in these cells should overcome the transcriptional deficiency due to an insufficient amount of Adr1p. If the level of *ADH2* expression in *bcy1* mutant cells is the same as in wild-type cells, cAPK must be acting before Adr1p is required. If the level of *ADH2* expression is lower, cAPK may be acting either at or subsequent to the requirement for Adr1p. ADH native-gel analysis of extracts from *bcy1* mutant cells carrying several different *ADR1* expression plasmids showed that *ADH2* was being expressed (Fig. 7A, compare *ADR1*, *bcy1* and *CUP1*, *bcy1*). This observation is consistent with decreased *ADR1* expression playing a role in the loss of *ADH2* expression. *ADH2* expression, however, was significantly lower than in wild-type cells (Fig. 7A, compare *ADR1*, WT and *ADR1*, WT) having a similar level of Adr1p (Fig. 7B). This suggests that cAPK may also be acting at or subsequent to the requirement for Adr1p.

FIG. 7. Effect of Adr1p overexpression on the inhibition of *ADH2* expression by unregulated cAPK activity. Native and denatured whole-cell extracts were prepared from derepressed CMY215 Δ 1 cells containing the high-copy-number *ADR1* expression plasmids YEpI323*ADR1*, pKD43, and pKD44, and a high-copy-number control plasmid pKD9, which lacks the *ADR1* open reading frame (—). YEpI323*ADR1* carries the complete *ADR1* gene (25), while pKD43 and pKD44 carry fusions of the *ADR1* open reading frame to the *ADH1* and *CUP1* promoters, respectively. (A) ADH native gel showing the level of *ADH2* expression in cells containing the *ADR1* expression plasmids. The amount of ADHII activity relative to the amount found in CMY215 Δ 1 containing YEpI323*ADR1* is listed below each lane. (B) Adr1p Western blot showing the level of Adr1p in cells containing the *ADR1* expression plasmids. The range of protein amount loaded was 1.25 to 5 μ g. Relative levels of Adr1p are listed beneath each set of lanes as a percentage of that found in CMY215 Δ 1 containing YEpI323*ADR1*. nd, not detectable.

DISCUSSION

Previous studies have shown that unregulated cAPK activity inhibits *ADH2* expression and suggested that an Adr1p-dependent event involved in transcriptional initiation is the target. In this paper, we provide new supporting evidence indicating that Adr1p-dependent transcription is inhibited. *ADH2* mRNA did not accumulate and expression of an *ADH2-lacZ* reporter gene was not activated in *bcy1* mutant cells. In addition, expression driven by a heterologous UAS1-*CYC1* promoter was strongly inhibited. Taken together with the observation that expression driven by an *ADH2* promoter with an exact deletion of UAS1 is not significantly affected (17), this indicates that cAPK acts through UAS1 independently of other *ADH2* promoter elements. Since Adr1p binds to UAS1 with high affinity and no other positively acting factors that bind to UAS1 have been identified biochemically or genetically, Adr1p or an Adr1p-dependent process is the most likely target of cAPK.

Our data indicate that one way cAPK acts through UAS1 is by decreasing the abundance of Adr1p. Since both wild-type and Adr1-5^c protein were similarly affected, phosphorylation of serine 230 does not appear to be important for this effect. It has been suggested that inhibition of *ADH2* expression by cAPK also does not involve phosphorylation of serine 230 (17, 19). Instead, decreased abundance of Adr1p appears to be a more important factor. This also provides an explanation for the *TPK* allele-specific inhibition of *ADH2* expression reported for *bcy1* mutant cells in derepressing medium (12, 19). The amount of cAPK activity in each *bcy1 tpk* mutant strain is determining the level of Adr1p which, if below the required threshold, is unable to support a wild-type level of *ADH2* expression. In strains with only the wild-type *TPK1* or *TPK2* genes, *ADH2* expression is inhibited to a much greater extent than in a strain with only *TPK3*. Since the *TPK3* gene is weakly

expressed compared to the *TPK1* and *TPK2* genes (33), a strain with only the *TPK3* gene should have a lower level of cAPK activity. Therefore, the level of Adr1p in a strain with only the *TPK3* gene should be higher than in strains with only the *TPK1* or *TPK2* genes.

The decreased abundance of Adr1p resulted from inhibition of *ADR1* transcription. Sequences between bp -555 and -207 in the *ADR1* promoter were required for this effect. However, a discrete element within this region did not appear to be the primary mediator of inhibition. All promoters containing a portion of the sequence between bp -453 and -207 were still subject to inhibition. Additionally, the sequences that mediated inhibition were not identical in repressed and derepressed cells. Sequences between bp -555 and -453 appeared to mediate inhibition in derepressed cells but not in repressed cells. Any conclusions derived from these observations must be tempered by the fact that they were derived exclusively from experiments involving a small set of promoter deletions. With this in mind, it seems likely that cAPK does not act through a single transcription factor that binds to the *ADR1* promoter as it does with Adr1p to inhibit *ADH2* expression. Rather, cAPK may act through several factors, and at least one of these may play a role in glucose-regulated transcriptional activation.

The *SAF* gene products are potential targets of cAPK. These proteins are positively acting factors required for the maintenance of *ADR1* expression in repressed cells (14). Mutations in the *SAF* genes decrease the level of *ADR1* expression by approximately the same amount as unregulated cAPK activity in repressed cells. Also like unregulated cAPK activity, these mutations suppress *ADH2* expression in repressed cells with the *ADR1-5^c* allele. In addition, *saf* mutations and cAPK both act through sequences 5' to the translational start site of the *ADR1* gene. These mutations, however, affect *ADR1* expression only in repressed cells. Therefore, the *Saf* proteins probably do not play a significant role in the cAPK-dependent inhibition observed in derepressed cells. Further work will be required to identify the function of the *Saf* proteins and to determine whether cAPK significantly affects this function.

The potential importance of transcription factor levels in determining the level of target gene expression was illustrated by Griggs and Johnston (23). They showed that a 5-fold decrease in *GAL4* expression reduced expression of the *GAL1* target gene by as much as 40-fold and that even a 2-fold decrease in *GAL4* expression significantly lowered the level of *GAL1* expression. Although the level of Adr1p can limit the derepressed level of *ADH2* expression, unlike for Gal4p, this occurs only at very low levels of the transcription factor. *ADR1* expression must be decreased by more than eightfold before the level of *ADH2* expression is even slightly reduced. This may reflect a difference both in the cellular levels of Adr1p and Gal4p and in their modes of binding to target promoters. Although the levels of Adr1p and Gal4p have never been directly compared, it would not be surprising if Adr1p were significantly more abundant than Gal4p based on the potential number of target genes. While Gal4p is thought to be required only for expression of galactose-regulated genes (29), Adr1p is required for expression of the *ADH2* gene (18), genes involved in glycerol catabolism (6, 35), and genes involved in peroxisomal biogenesis and function (39). Expression of the target genes also exhibited different forms of dependence on transcription factor levels. While the dependence of *GAL1* expression on *GAL4* expression was sigmoidal (23), perhaps reflecting the cooperative binding of Gal4p to the *GAL1* promoter, the dependence of *ADH2* expression on *ADR1* expression was not. This is consistent with the noncooperative binding of two

Adr1p monomers to UAS1 that has been demonstrated previously (45).

The weak dependence of *ADH2* expression on the level of Adr1p provides some insight into how glucose exerts negative control over *ADH2* expression. *ADR1* expression is subject to strain-dependent negative regulation by glucose (8). Our results suggest that in yeast strains where *ADR1* expression is regulated eightfold or less by glucose, the level of Adr1p present in repressed cells is sufficient to support a fully derepressed level of *ADH2* expression. This is consistent with a previous report showing that the level of *ADR1* expression is not the limiting factor that determines the level of *ADH2* expression in derepressed cells (16). However, in strains where *ADR1* expression is regulated to a greater extent, the amount of Adr1p does appear to be limiting. The expected level of *ADH2* expression in strains exhibiting 20-fold regulation by glucose is only 28% of the fully derepressed level as estimated from the data presented in Fig. 6. Therefore, decreased *ADR1* expression may play a small role in the repression of *ADH2* expression in some yeast strains. However, it is clearly insufficient to fully explain the stringent control exerted by glucose which completely blocks *ADH2* expression. This is consistent with a previous proposal that glucose repression occurs primarily through a combination of one or more other mechanisms (47).

Our data also suggest that decreasing the level of Adr1p is not the only mechanism by which cAPK inhibits *ADH2* expression. One alternate mechanism might involve the phosphorylation of Adr1p by cAPK and its subsequent inactivation. Although there is some indirect evidence suggesting that such phosphorylation may not affect the activity of Adr1p (19), this possibility has not been conclusively ruled out. Another mechanism might involve the inactivation of other participants in *ADR1*-dependent transcriptional activation. These include RNA polymerase II and holoenzyme-associated factors such as the Swi/Snf complex, TFIID, which is composed of TBP and TBP-associated factors, other basal transcription factors such as TFIIA and TFIIB, and the Ada2p/Ada3p complex (40). cAPK could potentially inhibit the activity of any one of these factors by phosphorylating its protein subunits. The amino acid sequences of over half of these proteins contain one or more motifs resembling a cAPK phosphorylation site (20). It has been reported that underproduction of the largest subunit of polymerase II is not lethal and causes promoter-specific defects in expression (1). Depletion of Toa2p, the 13.5-kDa subunit of TFIIA, also does not appear to be lethal and reduces the level of polymerase II-dependent transcription in vivo (27). These observations argue that it would be possible for cAPK to inhibit the expression of specific genes by reducing the abundance of individual components of the basal transcription apparatus. Whether the minor reduction in the level of Sua7p observed in *bcy1* mutant cells in combination with the large decrease in Adr1p abundance is capable of blocking *ADH2* expression is not clear.

Adr1p has been commonly cited as an example of a transcription factor whose activity is negatively controlled by cAPK-dependent phosphorylation. The experiments presented here suggest that this negative control is exerted through several mechanisms, one being the inhibition of *ADR1* transcription, which act in concert to inhibit the activity of Adr1p.

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