

## *ADR1*<sup>c</sup> Mutations Enhance the Ability of ADR1 To Activate Transcription by a Mechanism That Is Independent of Effects on Cyclic AMP-Dependent Protein Kinase Phosphorylation of Ser-230†

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Four *ADR1*<sup>c</sup> mutations that occur close to Ser-230 of the *Saccharomyces cerevisiae* transcriptional activator ADR1 and which greatly enhance the ability of ADR1 to activate *ADH2* expression under glucose-repressed conditions have been shown to reduce or eliminate cyclic AMP-dependent protein kinase (cAPK) phosphorylation of Ser-230 in vitro. In addition, unregulated cAPK expression in vivo blocks *ADH2* derepression in an ADR1-dependent fashion in which *ADR1*<sup>c</sup> mutations display decreased sensitivity to unregulated cAPK activity. Taken together, these data have suggested that *ADR1*<sup>c</sup> mutations enhance ADR1 activity by blocking cAPK phosphorylation and inactivation of Ser-230. We have isolated and characterized an additional 17 *ADR1*<sup>c</sup> mutations, defining 10 different amino acid changes, that were located in the region defined by amino acids 227 through 239 of ADR1. Three observations, however, indicate that the *ADR1*<sup>c</sup> phenotype is not simply equivalent to a lack of cAPK phosphorylation. First, only some of these newly isolated *ADR1*<sup>c</sup> mutations affected the ability of yeast cAPK to phosphorylate corresponding synthetic peptides modeled on the 222 to 234 region of ADR1 in vitro. Second, we observed that strains lacking cAPK activity did not display enhanced *ADH2* expression under glucose growth conditions. Third, when Ser-230 was mutated to a nonphosphorylatable residue, lack of cAPK activity led to a substantial increase in *ADH2* expression under glucose-repressed conditions. Thus, while cAPK controls *ADH2* expression and ADR1 is required for this control, cAPK acts by a mechanism that is independent of effects on ADR1 Ser-230. It was also observed that deletion of the *ADR1*<sup>c</sup> region resulted in an *ADR1*<sup>c</sup> phenotype. The *ADR1*<sup>c</sup> region is, therefore, involved in maintaining ADR1 in an inactive form. *ADR1*<sup>c</sup> mutations may block the binding of a repressor to ADR1 or alter the structure of ADR1 so that transcriptional activation regions become unmasked.

The regulation of the glucose-repressible alcohol dehydrogenase (ADH II, encoded by the *ADH2* gene) from *Saccharomyces cerevisiae* is mediated by several pathways. The *SNF1* protein kinase and the *REG1* gene constitute one pathway (10, 35), but their site of action at *ADH2* has not been identified. The transcriptional activator ADR1 acts independently of *SNF1* (10) and binds to the upstream activation sequence, UAS1, located between bp -271 and -291 of the *ADH2* promoter (39). Part of the glucose regulation of *ADH2* is mediated by control of ADR1 translation (37a). A third regulatory network whose factors have not been identified may act through UAS2, which is just upstream of UAS1 (39).

The importance of ADR1 in the glucose control of *ADH2* is evidenced by a class of *ADR1* mutations (*ADR1*<sup>c</sup>) causing enhanced *ADH2* transcription under repressed conditions (8, 11). These mutations do not affect ADR1 RNA (12) or protein levels (37a) and must activate ADR1 by a posttranslational mechanism. Four *ADR1*<sup>c</sup> mutations have been identified and found to occur between amino acids 228 and 231 of ADR1 in a putative cyclic AMP-dependent protein kinase (cAPK) phosphorylation consensus sequence (RRASF, where Ser-230 is the phosphoacceptor) (6, 12). ADR1 has been found to be a substrate for cAPK in vitro; cAPK phosphorylates both Ser-230 and some other site to the

N-terminal side of Ser-230 (6). The *ADR1*<sup>c</sup> mutations were shown to decrease or eliminate cAPK phosphorylation of ADR1 at Ser-230 in vitro. These data suggest that in *S. cerevisiae* the *ADR1*<sup>c</sup> alterations enhance ADR1 activity by interfering with the cAPK phosphorylation of Ser-230.

Subsequent genetic analysis indicated that unregulated cAPK activity, the result of disrupting the *BCY1* cAPK regulatory gene, reduces *ADH2* expression (6). The effects of the *bcy1* disruption were relieved, albeit incompletely, by *ADR1*<sup>c</sup> mutations (6). These results provided support for the model that cAPK inactivated ADR1 function by phosphorylating Ser-230, although Ser-230 appeared not to be the only site of cAPK inactivation. More recently it has been demonstrated that all of the effects of cAPK on *ADH2* expression are mediated by ADR1 (10). Two other protein kinases, *SNF1* and *SCH9*, were observed to affect *ADH2* expression independently of both ADR1 and cAPK (10).

In this study, we have characterized 17 additional *ADR1*<sup>c</sup> alleles with the expectation of furthering our understanding of how these mutations activate ADR1. Using synthetic peptides, we observed that not all *ADR1*<sup>c</sup> mutations affected the cAPK phosphorylation of Ser-230 in vitro. More importantly, lack of cAPK activity did not elicit an *ADR1*<sup>c</sup> phenotype in vivo. These data argue for a model in which *ADR1*<sup>c</sup> mutations alter the structure and function of ADR1 independent of cAPK phosphorylation of Ser-230. In addition, when Ser-230 could not be phosphorylated, a lack of cAPK was observed to result in enhanced ADR1 function. Accordingly, cAPK must act through another site on ADR1 or through another protein that mediates ADR1 activity.

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TABLE 1. Yeast strains

Strain	Genotype
43-2B	<i>MAT<math>\alpha</math> adh1-11 adh3 ural his4</i>
410-7a	<i>MAT<math>\alpha</math> adh1-11 leu2 ade8 ura3 his3 trp1 tpk2::HIS3 tpk3::TRP1</i>
S13-58ArA	<i>MAT<math>\alpha</math> his3 leu2 ura3 trp1 ade8 tpk1w1 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>
409-1a	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 ade8 trp1 leu2 tpk1::URA3 tpk2::HIS3</i>
441-2-4b	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 ade8 tpk2::HIS3 tpk1w1</i>
439-3a	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 tpk1::URA3 tpk3::TRP1</i>
438-8a	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 ade8 tpk1::URA3 tpk3::TRP1</i>
387-5d	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 ade8 tpk2::HIS3 tpk3::TRP1</i>
442-7a	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 tpk1::URA3 tpk2::HIS3 tpk3w1</i>
410-1c	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 tpk1::URA3 tpk3::TRP1</i>
409-7c	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 ade8 tpk1::URA3 tpk2::HIS3</i>
639-3b	<i>MAT<math>\alpha</math> adh1-11 ADR1-7<math>^c</math> adh3 ura3 his3 trp1 leu2 tpk1::URA3 tpk2::HIS3</i>
630-4d	<i>MAT<math>\alpha</math> adh1-11 adh3 cre1-1 ura3 his3 trp1 leu2 tpk1::URA3 tpk2::HIS3</i>
315-1D	<i>MAT<math>\alpha</math> adh1-1 trp1 ural</i>
482-2b	<i>MAT<math>\alpha</math> adh1-11 ura3 his3 trp1 leu2 tpk2::HIS3 tpk3::TRP1</i>

## MATERIALS AND METHODS

**Yeast strains.** Yeast strains are listed in Table 1. Strains containing the *ADR1* alleles 9<sup>c</sup> through 25<sup>c</sup> were isogenic to 315-1D (32).

**Identifying *ADR1*<sup>c</sup> mutations.** Strains containing dominant mutations that allowed *ADH2* expression under glucose growth conditions were obtained by V. Williamson in the course of a previous study (32). Each mutant was an independent spontaneous isolate and was shown not to result from Ty insertions at either the *ADH2* or *ADH4* locus (32). Tetrad analysis was used to determine whether the dominant mutations mapped to the *ADR1* locus. *ADR1*<sup>c</sup> alleles were rescued from *S. cerevisiae* and localized to the region between bp +440 and +1076 as described previously (6, 12). For each different *ADR1*<sup>c</sup> allele, the region between bp +440 and +1076 was sequenced to identify the *ADR1*<sup>c</sup> lesion.

**cAPK phosphorylation of synthetic *ADR1* peptides.** Peptides were synthesized and isolated as described previously (13). The yeast TPK1 (37) enzyme was provided by M. Zoller, and the porcine heart cAPK was provided by S. Taylor. Conditions for determining the *K<sub>m</sub>* of phosphorylation of each peptide were as described previously (13). The unique site of phosphorylation for each peptide, except *ADR1*-222-234 (F231S), was determined to be Ser-230 by chymotryptic analysis as described previously (13). Peptide *ADR1*-222-234 (F231S) was found to be stoichiometrically phosphorylated on serine, but the precise site of phosphorylation was not determined.

**Growth conditions and *ADH* assays.** Conditions for growth of cultures on YEP medium (2% Bacto Peptone, 1% yeast extract, 20 mg of adenine and uracil per liter) have been described previously (15). *ADH* II enzyme activities were

TABLE 2. Identification of *ADR1*<sup>c</sup> mutations

<i>ADR1</i> <sup>c</sup> allele <sup>a</sup>	Base pair change	Nucleotide	Amino acid change <sup>b</sup>
<i>ADR1</i> -9 <sup>c</sup>	G to T	680	R227L
<i>ADR1</i> -10 <sup>c</sup>	G to A	683	R228K
<i>ADR1</i> -5 <sup>c</sup>	G to A	683	R228K
<i>ADR1</i> -8 <sup>c</sup>	G to C	685	A229P
<i>ADR1</i> -13 <sup>c</sup>	C to T	689	S230L
<i>ADR1</i> -19 <sup>c</sup>	C to T	689	S230L
<i>ADR1</i> -23 <sup>c</sup>	C to T	689	S230L
<i>ADR1</i> -7 <sup>c</sup>	C to T	689	S230L
<i>ADR1</i> -2 <sup>c</sup>	T to C	692	F231S
<i>ADR1</i> -4 <sup>c</sup>	T to C	692	F231S
<i>ADR1</i> -15 <sup>c</sup>	C to G	696	S232R
<i>ADR1</i> -20 <sup>c</sup>	C to G	696	S232R
<i>ADR1</i> -25 <sup>c</sup>	C to G	696	S232R
<i>ADR1</i> -21 <sup>c</sup>	C to A	696	S232R
<i>ADR1</i> -24 <sup>c</sup>	C to A	696	S232R
<i>ADR1</i> -22 <sup>c</sup>	G to T	695	S232I
<i>ADR1</i> -11 <sup>c</sup>	C to G	698	A233G
<i>ADR1</i> -14 <sup>c</sup>	G to A	697	A233T
<i>ADR1</i> -16 <sup>c</sup>	A to G	716	Y239C
<i>ADR1</i> -17 <sup>c</sup>	A to G	716	Y239C
<i>ADR1</i> -18 <sup>c</sup>	T to A	715	Y239N

<sup>a</sup> *ADR1*-5<sup>c</sup> is identified in reference 12, and *ADR1*-7<sup>c</sup>, *ADR1*-2<sup>c</sup>, and *ADR1*-4<sup>c</sup> are identified in reference 6.

<sup>b</sup> Amino acid changes are given in the format A229P, where A represents the residue in the *ADR1* gene, the number refers to the residue, and P refers to the residue encoded by the *ADR1*<sup>c</sup> allele.

assayed as previously described (10, 15). Yeast transformations were conducted by the LiAcetate procedure (22).

**Gene disruptions and constructions.** The *BCY1* gene disrupted with the *LEU2* gene was used as previously described (36). The *tpk2w11*, the *tpk1w10*, and the *tpk3w11* through -14 alleles were specifically selected as suppressors of *bcy1*-induced phenotypes (inability to grow at 30°C after a shift to 55°C for 1 h, dark colony color, and reduced growth on nonfermentative carbon sources) as previously described (5, 31). Genetic analysis was conducted as previously described (5) to verify that a *tpkw* allele was responsible for suppressing the unregulated cAPK activity caused by the *bcy1* allele. cAPK enzyme activity was determined as described previously (36).

*ADR1*-220/262 was constructed as follows. The *XmnI*-*SacI* fragment of *ADR1* (bp 786 to 1713) was ligated into pUC18 at the *SmaI* (blunt-ended) and *SacI* sites to generate plasmid LBp45. The *HindIII*-*ScaI* fragment of YRp7-*ADR1*-23A (12), extending from 1.5 kbp upstream of *ADR1* to bp +661, was subsequently ligated into LBp45 restricted with *HindIII* and *Sall* (filled in with a Klenow fragment). The resultant plasmid, LBp8, contained the 5' region of *ADR1* and the region encoding *ADR1* amino acids 1 to 220 placed in frame to residues 262 to 571. Between residues 220 and 262 were seven additional amino acid residues derived from the pUC18 polylinker (FDSRGSP). LBp8, after addition of the *TRP1* gene, was targeted for integration at the *adr1-1* locus in strain 500-16 following cleavage with *NruI* at bp 1517. Identification of integrants by genetic and Southern analysis has been described previously (2). Integration of the plasmid LBp8 (*TRP1*) at the *adr1-1* locus regenerates a complete *ADR1*-220/262 allele as well as a truncated *adr1-1* allele. *adr1-1* does not express a functional *ADR1* protein, since it contains a nonsense mutation in its 11th codon (3).

TABLE 3. ADH II enzyme activities in strains carrying *ADRI<sup>c</sup>* mutations

<i>ADRI<sup>c</sup></i> allele	Amino acid change	ADH II activity (mU/mg) <sup>a</sup>	
		Glucose	Ethanol
<i>ADRI</i>	None	0 ± 0	1,300 ± 160
<i>ADRI-9<sup>c</sup></i>	R227L	110 ± 8.0	3,300 ± 460
<i>ADRI-10<sup>c</sup></i>	R228K	140 ± 4.8	3,200 ± 490
<i>ADRI-8<sup>c</sup></i>	A229P	360 ± 12	3,900 ± 360
<i>ADRI-13<sup>c</sup></i>	S230L	140 ± 14	4,200 ± 430
<i>ADRI-15<sup>c</sup></i>	S232R	160 ± 4.1	3,200 ± 590
<i>ADRI-22<sup>c</sup></i>	S232I	160 ± 16	2,400 ± 680
<i>ADRI-11<sup>c</sup></i>	A233G	69 ± 2.7	3,400 ± 630
<i>ADRI-14<sup>c</sup></i>	A233T	110 ± 2.5	3,800 ± 540
<i>ADRI-16<sup>c</sup></i>	Y239C	260 ± 8.7	2,800 ± 760
<i>ADRI-18<sup>c</sup></i>	Y239N	120 ± 12	3,000 ± 360
<i>ADRI-220/262</i>		160 ± 33	5,900 ± 800

<sup>a</sup> ADH II enzyme activities were determined following overnight on YEP medium supplemented with either 8% glucose or 3% ethanol. All strains except that containing *ADRI-220/226* are isogenic to 315-1D (*ADR1*). ADH values (± standard error of the mean) represent the averages of at least four separate determinations. The *ADRI-220/262* allele is present in strain 500-16, which, when containing the wild-type *ADRI* gene, displays ADH II activities of 5 and 2,000 mU/mg under glucose and ethanol growth conditions, respectively (9).

## RESULTS

**Identification of 17 new *ADRI<sup>c</sup>* mutations.** Twenty independently isolated strains containing dominant mutations which allowed glucose-insensitive *ADH2* expression and which were not the result of Ty transposition at either the *ADH2* or *ADH4* locus (32) were analyzed. Tetrad analysis indicated that 18 of these contained mutations at the *ADRI* locus (*ADRI<sup>c</sup>*) and 2 carried mutations at *ADH2* (data not shown). Of the *ADRI<sup>c</sup>* alleles, 17 were rescued from *S. cerevisiae*, and the *ADRI<sup>c</sup>* lesions were localized and sequenced. All 17 mutations were found to result from single base pair changes (Table 2). Ten different amino acid changes that defined eight new *ADRI<sup>c</sup>* mutations (R228K and S230L had been obtained previously) (6, 12) that substitute amino acids in the region of residues 227 to 239 of *ADR1* (Fig. 1) were identified. These new mutations occurred in the same region of *ADR1* as did the previous four *ADRI<sup>c</sup>* mutations that surround Ser-230 (6, 12). The occurrence of all 21 known *ADRI<sup>c</sup>* mutations in the region containing Ser-230 suggests that this is the only domain of *ADR1* which can be mutated to allow *ADR1* to bypass the effects of glucose repression.

The effect on *ADH2* expression of each of the different *ADRI<sup>c</sup>* mutations is given in Table 3. The mutations resulted in ADH II enzyme activity levels ranging from 70 to 360 mU/mg under glucose growth conditions. Under ethanol growth conditions, each of the mutations resulted in similar ADH II enzyme activity levels (about 3,500 mU/mg). The observation that under glucose growth conditions *ADRI-8<sup>c</sup>* (A229P [i.e., on A-to-P change at position 229]) and *ADRI-16<sup>c</sup>* (Y239C) displayed enhanced *ADR1* function relative to *ADRI-13<sup>c</sup>* (S230L) suggests that structural alterations in the *ADRI<sup>c</sup>* region are more important to enhancing *ADR1* function than a simple lack of the potential for cAPK phosphorylation of Ser-230 (as in *ADRI-13<sup>c</sup>*).

**Only some *ADRI<sup>c</sup>* mutations affect cAPK phosphorylation of synthetic *ADR1* peptides.** Our previous analysis indicated that *ADRI<sup>c</sup>*-R228K and *ADRI<sup>c</sup>*-F231S reduced the phosphorylation by cAPK of *ADR1* Ser-230 in vitro (6). This was expected on the basis of other studies that defined the substrate recognition determinants of cAPK (4, 24). Several

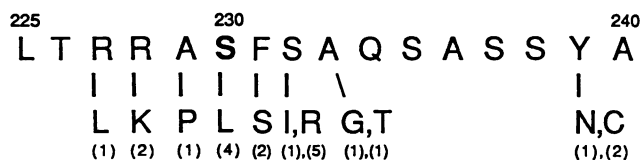


FIG. 1. Amino acid changes corresponding to *ADRI<sup>c</sup>* mutations. The amino acid changes for the *ADRI<sup>c</sup>* mutations are indicated below the *ADR1* sequence that extends from amino acids 227 to 240. The number below the amino acid change indicates the number of independently isolated alleles giving rise to that change. The numbers above the *ADR1* sequence refer to the *ADR1* protein sequence.

of the newly identified *ADRI<sup>c</sup>* mutations appeared unlikely, however, to similarly affect cAPK phosphorylation. It is known that some mammalian cAPK substrates, e.g., the CREB protein (19) and fructose-1,6-bis-phosphatase (17), are phosphorylated effectively at the sequence RRPS, a sequence identical to that found in *ADR1<sup>c</sup>*-A229P (Table 3). Also, it has been shown that a proline at the -1 position (relative to the phosphoacceptor site designated 0) has little or no effect on phosphorylation of synthetic peptide substrates (7, 13, 17, 20). In addition, the yeast cAPK regulatory subunit BCY1, a known in vivo substrate for yeast cAPK, contains a glycine residue in its +3 position, resulting in a sequence (RRASVSG) (36) which is very similar to that of *ADR1-11<sup>c</sup>*-A233G (RRASFSG) (Table 4). We therefore examined whether the *ADRI<sup>c</sup>* mutations were likely to affect cAPK phosphorylation by using synthetic peptides modeled on the sequence containing Ser-230 of *ADR1*.

Synthetic peptides have generally been found to be excellent substrates for mammalian cAPK and to be phosphorylated with kinetics similar to those of the natural protein substrates (24). Moreover, synthetic peptides modeled on the *ADR1* sequence at Ser-230 have previously been shown to be excellent substrates for yeast cAPK (TPK1 isozyme) (13). *ADR1-222-234*, which is phosphorylated with a  $K_m$  of 6.8  $\mu$ M by the yeast TPK1 enzyme (Table 4), was chosen as our parent substrate (13). Eight different *ADR1* peptide analogs were synthesized, each containing an amino acid change corresponding to an *ADRI<sup>c</sup>* mutation, and the ability of each peptide to be phosphorylated by yeast cAPK was analyzed. Some *ADRI<sup>c</sup>* mutations causing alterations in previously known important recognition determinants of cAPK (e.g., R227L, R228K, F231S, and S232R) (4, 24) had very dramatic effects on cAPK phosphorylation of Ser-230. These alterations increased the  $K_m$  for phosphorylation by 20- to 400-fold (Table 4). These results are in agreement with our previous data demonstrating that R228K and F231S reduce the ability of cAPK to phosphorylate Ser-230 in vitro (6). In contrast, other *ADRI<sup>c</sup>* mutations had very little or no effect on cAPK phosphorylation (e.g., A229P, S232I, A233G, and A233T) (Table 4). In fact, we observed no correlation between cAPK recognition of the *ADRI<sup>c</sup>* peptide analogs and the ability of the *ADR1* or *ADRI<sup>c</sup>* protein to activate *ADH2* under glucose growth conditions (Table 3). These results do not appear to be due to some special recognition feature of the yeast TPK1 isozyme, since similar effects on Ser-230 phosphorylation were also obtained with the distantly related porcine cAPK (Table 4). In addition, the recently determined crystal structure of mouse cAPK clearly indicates that the A229P change would not be expected to affect cAPK phosphorylation (26). These data do not support the model that the only effect of the *ADRI<sup>c</sup>* mutations on *ADR1* function is to alter cAPK phosphorylation of Ser-230.

TABLE 4. Effect of *ADR1<sup>c</sup>* mutations on cAPK phosphorylation of ADR1 synthetic peptides

Peptide	Sequence	$K_m$ ( $\mu\text{M}$ ) <sup>a</sup>	
		Yeast cAPK	Mammalian cAPK
ADR1-222-234	LKKLRRASFSAQ	6.8 ± 0.85	0.26 ± 0.011
ADR1-R227L	LKKLTLRASFSQAQ	2,500 ± 140	170 ± 23
ADR1-R228K	LKKLTKASFSQAQ	230 ± 18	3.5 ± 0.70
ADR1-A229P	LKKLRRPFSFSQAQ	4.9 ± 0.86	0.34 ± 0.009
ADR1-F231S	LKKLRRASSSQAQ	144 ± 13	6.3 ± 0.88
ADR1-S232R	LKKLRRASFRAQ	140 ± 43	6.6 ± 1.7
ADR1-S232I	LKKLRRASFIAQ	15 ± 2.1	0.97 ± 0.18
ADR1-A233G	LKKLRRASFSGQ	7.3 ± 0.36	0.74 ± 0.19
ADR1-A233T	LKKLRRASFSTQ	9.0 ± 3.0	0.46 ± 0.051

<sup>a</sup> Peptide phosphorylation was determined as described in Materials and Methods. Kinetic constants ( $\pm$  standard error of the mean) are the averages of three to five separate determinations and were estimated by fitting the data to the Michaelis-Menten equation by the least-squares method.

For the remaining three *ADR1<sup>c</sup>* mutations, S230L, Y239C, and Y239N, cAPK did not phosphorylate the peptide 222-234 containing the S230L change (data not shown). We did not analyze peptides corresponding to the two alterations at Tyr-239, but it has been observed that yeast cAPK phosphorylates ADR1 peptide 225-234 with a  $K_m$  of 13  $\mu\text{M}$ , a value that is only twice that found for ADR1 peptide 225-241 (7.0  $\mu\text{M}$ ) (13). Sequences from 235 to 241 appear, therefore, to have only a small effect on cAPK phosphorylation of Ser-230.

#### Lack of cAPK activity does not confer an *ADR1<sup>c</sup>* phenotype.

Because the results described above were conducted in vitro, we acknowledge the possibility that the *ADR1<sup>c</sup>* alterations could result in gross changes in the structure of the full-length ADR1 protein that would affect cAPK phosphorylation of Ser-230 in vivo. To address this question, we analyzed in vivo the effect of a lack of cAPK activity on *ADH2* expression. If the *ADR1<sup>c</sup>* phenotype were due solely to reducing cAPK phosphorylation of Ser-230, then strains containing no measurable cAPK activity would be expected to evince an *ADR1<sup>c</sup>* phenotype. We investigated this possibility by measuring the level of *ADH2* expression in strains containing deletions in two of the three *TPK* genes and a mutation in the third *TPK* gene (*tpkw* allele). Such strains lack measurable cAPK activity (reference 5 and Materials and Methods above) and are known to be refractory to the effects of disrupting the *BCY1* gene, a disruption that would otherwise cause unregulated cAPK activity (5). Our analysis of strains lacking cAPK activity indicated that the levels of ADH II expression under glucose growth conditions were unaltered from those found in wild-type strains (Table 5). These results were observed regardless of the combination of deleted and mutated *TPK* alleles (Table 5 and data not shown). Under ethanol growth conditions, however, lack of cAPK activity relieved the reduction in *ADH2* expression caused by unregulated cAPK activity (e.g., compare strains isogenic to 410-1c [Table 5]), indicating that strains containing disruptions in two *TPK* genes and a mutation in the third behaved as though they lacked cAPK activity.

While it can be argued that under glucose growth conditions trace levels of cAPK activity that fall below our detection capabilities may persist and be sufficient to maintain glucose repression of ADR1, several observations suggest that this is not the case. First, similar strains lacking detectable cAPK activity in an *ADR1-7<sup>c</sup>* background (con-

TABLE 5. Effect of lack of cAPK activity on *ADH2* expression<sup>a</sup>

Relevant genotype	Background strain	ADH II activity (mU/mg)	
		Glucose	Ethanol
<i>TPK1 TPK2 TPK3<sup>b</sup></i>	43-2B	8	2,500
<i>TPK1 tpk2 tpk3</i>	410-7a	8.3 ± 1.4	3,700 ± 380
<i>TPK1 tpk2 tpk3 bcy1</i>	410-7a	8.7 ± 0.99	430 ± 89
<i>tpk1w1 tpk2 tpk3 bcy1<sup>c</sup></i>	Segregants	9.6 (1–21)	4,100 (2,200–5,200)
<i>tpk1 TPK2 tpk3</i>	410-1c	5.3 ± 1.6	4600 ± 480
<i>tpk1 TPK2 tpk3 bcy1</i>	410-1c	5.0 ± 2.1	260 ± 50
<i>tpk1 tpk2w11 tpk3 bcy1</i>	410-1c	4.0 ± 0.81	4,500 ± 930
<i>tpk1w1 tpk2 tpk3<sup>d</sup></i>	Segregants	2.2 (1–4)	2,600 (1,700–3,300)
<i>tpk1 tpk2w11 tpk3<sup>e</sup></i>	Segregants	5.0 (1–18)	3,000 (1,200–5,400)
<i>tpk1 tpk2 tpk3<sup>e</sup></i>	Segregants	0.7 (0–2.1)	6,900 (6,500–7,200)

<sup>a</sup> ADH II enzyme activities were determined as described in Table 3, footnote a. Strains derived from the same background strain are isogenic, except for the disrupted allele indicated in the table. The *tpk1w1* allele has been previously described (5). The *tpk2w11* allele was obtained as described in Materials and Methods. For individual strains, the values represent the averages ( $\pm$  standard error of the mean) of at least six separate determinations. For segregants, at least three segregants of each genotype were assayed and their values were averaged (the range of values is given in parentheses).

<sup>b</sup> Values taken from reference 14.

<sup>c</sup> Segregants from the cross S13-58ArA × 409-1a.

<sup>d</sup> Segregants from the crosses 441-2-4b × 439-3a and 441-2-4b × 438-8a.

<sup>e</sup> Segregants from the cross 387-5d × 442-7a.

taining a nonphosphorylatable residue at Ser-230) resulted in a 4- to 15-fold enhancement of *ADH2* expression under glucose growth conditions (see below) (Table 6). Second, strains which contain the *tpkw1* allele and the disrupted *tpk2* and *tpk3* alleles have been found to be defective in glucose repression of mitochondrial functions (30). Moreover, the *CTT1* gene (encoding catalase T) displays elevated levels of expression under glucose growth conditions in a *tpkw1 tpk2 tpk3* background (1). These three observations suggest that there is insufficient cAPK activity in strains carrying a *tpkw* allele to maintain completely the effects of glucose repression. Finally, we have observed that strains containing disruptions in all three *TPK* alleles (presumably viable because of the presence of another mutation, such as *yak1*) (16) were fully glucose repressed for *ADH2* expression (Table 5). Our results indicate that lack of cAPK activity does not confer an *ADR1<sup>c</sup>* phenotype and that the *ADR1<sup>c</sup>* mutations do not enhance ADR1 function by interfering with cAPK phosphorylation of Ser-230. It remains possible that Ser-230 is phosphorylated by a protein kinase other than cAPK and that the *ADR1<sup>c</sup>* mutations interfere with the role of this enzyme.

#### cAPK inactivates ADR1 through a site other than Ser-230.

Because cAPK phosphorylation of Ser-230 cannot be solely responsible for maintaining ADR1 in an inactive state, we surmised that the effects of cAPK may be observed only when ADR1 is in an activated form such as that which occurs under ethanol growth conditions. Factors other than decreased cAPK phosphorylation would contribute to the activation of ADR1 under derepressed conditions. To test this idea, we repeated the experiment described above with

TABLE 6. Effect of lack of cAPK activity in combination with the *ADRI-7<sup>c</sup>* allele<sup>a</sup>

Relevant genotype	Background strain	ADH II activity (mU/mg)	
		Glucose	Ethanol
<i>tpk1 tpk2 TPK3 ADRI-7<sup>c</sup></i>	409-7c	38 ± 3.4	11,000 ± 860
<i>tpk1 tpk2 TPK3 ADRI-7<sup>c</sup> bcy1</i>	409-7c	14 ± 0.9	8,700 ± 740
<i>tpk1 tpk2 tpk3w11 ADRI-7<sup>c</sup> bcy1</i>	409-7c	590 ± 12	20,000 ± 2,500
<i>tpk1 tpk2 tpk3w11 ADRI-7<sup>c</sup> bcy1 pBCY1<sup>b</sup></i>	409-7c	400 ± 46	ND
<i>tpk1 tpk2 TPK3 ADRI-7<sup>c</sup></i>	639-3b	110 ± 8.0	ND
<i>tpk1 tpk2 TPK3 ADRI-7<sup>c</sup> bcy1</i>	639-3b	65 ± 5.7	ND
<i>tpk1 tpk2 tpk3w12 ADRI-7<sup>c</sup> bcy1</i>	639-3b	870 ± 99	ND
<i>tpk1 tpk2 TPK3 ADRI-5<sup>c</sup></i>	409-7c	120 ± 23	7,800 ± 830
<i>tpk1 tpk2 TPK3 ADRI-5<sup>c</sup> bcy1</i>	409-7c	34 ± 5.5	9,000 ± 610
<i>tpk1 tpk2 tpk3w14 bcy1 ADRI-5<sup>c</sup></i>	409-7c	330 ± 40	20,000 ± 1,500
<i>TPK1 tpk2 tpk3 ADRI-7<sup>c</sup></i>	482-2b	51 ± 14	14,000 ± 1,100
<i>TPK1 tpk2 tpk3 ADRI-7<sup>c</sup> bcy1</i>	482-2b	56 ± 5.5	5,000 ± 690
<i>tpk1w10 tpk2 tpk3 ADRI-7<sup>c</sup> bcy1</i>	482-2b	190 ± 26	23,000 ± 290
<i>tpk1 tpk2 TPK3 cre1-1</i>	630-4d	190 ± 14	ND
<i>tpk1 tpk2 TPK3 cre1-1 bcy1</i>	630-4d	140 ± 13	ND
<i>tpk1 tpk2 tpk3w13 cre1-1 bcy1</i>	630-4d	200 ± 24	ND

<sup>a</sup> ADH II enzyme activities were determined as described in Table 3, footnote a. The *tpk1w10* and *tpk3w11*, *tpk3w12*, *tpk3w13*, and *tpk3w14* alleles were identified as described in Materials and Methods. Strains derived from the same background strain are isogenic, except for the disrupted and mutated *tpkw* alleles as indicated. 409-7c strains containing the *ADRI-7<sup>c</sup>* and *ADRI-5<sup>c</sup>* alleles were derived by integration of plasmids YRp7-*ADRI-7<sup>c</sup>*-23A and YRp7-*ADRI-5<sup>c</sup>*-23A, respectively, into strain 409-7c (6). ND, not determined.

<sup>b</sup> Plasmid YEp24-*BCY1* that contains the full-length *BCY1* gene (36) was transformed into the parental strain 409-7c-1-w11 (*tpk3w11 bcy1*). In order to select for YEp24-*BCY1* transformants, isolates of strain 409-7c-1-w11 that contained a defect in the *URA3* gene were first identified following exposure to 5-fluoro-orotic acid (38).

strains carrying the *ADRI-7<sup>c</sup>* allele. We presumed that the *ADRI-7<sup>c</sup>* (S230L) mutation causes a conformational change in ADR1 that may mimic ADR1 structure, either partially or completely, under ethanol growth conditions. In such a case, the effect of a lack of cAPK activity on ADR1 activity and *ADH2* expression might become discernible. Strain 409-7c carrying the *ADRI-7<sup>c</sup>* allele and a single functional *TPK* gene (*TPK3*) was analyzed (Table 6). *ADH2* expression decreased about twofold under glucose growth conditions when the *BCY1* gene was disrupted (6) (Table 6). When the remaining *TPK* gene was mutated (allele *tpk3w11*) to generate a yeast strain containing no measurable cAPK activity (data not shown), *ADH2* expression increased to a level that was 15-fold higher than that found in the parental strain containing a functional *TPK3* allele. *ADH2* expression also increased under ethanol growth conditions (Table 6). A similar increase (eightfold) in *ADH2* expression under glucose growth conditions was obtained with another strain carrying the *ADRI-7<sup>c</sup>* allele and lacking cAPK activity as demonstrated with a second set of isogenic strains (639-3b strains [Table 6]), confirming that these results are not strain depen-

dent. These results are not specific to these *tpk3w* alleles, since eight additional independently isolated *tpk3w* alleles in strain 639-3b resulted in ADH II enzyme levels under glucose growth conditions that ranged from 730 to 950 mU/mg. We also tested whether the *ADRI-5<sup>c</sup>* allele (R228K) behaved in a similar manner. A strain lacking cAPK activity in an *ADRI-5<sup>c</sup>* background expressed threefold more ADH II activity under glucose growth conditions and twofold more under ethanol conditions than an isogenic strain containing cAPK activity (Table 6). These observations are also not limited to *tpk3w* alleles, since similar results were obtained in an *ADRI-7<sup>c</sup>*-containing strain that carried *tpk2* and *tpk3* disruptions and a *tpk1w* allele (Table 6). In addition, we tested whether the *bcy1* disruption was influencing the high activities under glucose growth conditions by transforming strain 409-7c, which lacked cAPK activity, with a plasmid containing the *BCY1* gene (36). Elevated levels of *ADH2* expression were still observed in a strain with the *ADRI-7<sup>c</sup>* allele that lacked cAPK activity but contained a functional *BCY1* gene (Table 6).

In order to examine whether the effects described above were the result of the *ADRI<sup>c</sup>* alleles or were caused by a general increase in *ADH2* transcription, the effect of a lack of cAPK activity on a strain carrying the *cre1* allele was also analyzed. *cre1* mutations under glucose growth conditions enhance *ADH2* transcription in an ADR1-independent manner to a level similar to that observed with an *ADRI-7<sup>c</sup>* allele (14). Deletion of the *BCY1* gene in a *cre1* background resulted in a 25% reduction in *ADH2* expression under glucose growth conditions compared with that of the parental strain (Table 6). When the *TPK3* allele was also mutated, a return to a level of *ADH2* expression commensurate with that observed in the strain containing cAPK activity was obtained (Table 6). These results are essentially the same as that obtained with a strain carrying the wild-type *CRE1* and *ADRI* genes (Table 5). The enhanced *ADH2* expression that is observed with lack of cAPK in an *ADRI-7<sup>c</sup>* background is, therefore, not the result of a general elevated level of *ADH2* transcription. Instead, it is dependent on the *ADRI<sup>c</sup>* allele. These results suggest that cAPK inactivates *ADH2* transcription through a site separate from Ser-230 of ADR1 and that the structure of the region around Ser-230 (as in an *ADRI-7<sup>c</sup>* allele) potentiates this effect of cAPK on *ADH2* expression.

#### Deletion of the *ADRI<sup>c</sup>* region confers an *ADRI<sup>c</sup>* phenotype.

The results described above suggest a lack of correlation between *ADRI<sup>c</sup>* mutations and cAPK phosphorylation of Ser-230 but do not reveal the importance of the sequence of the *ADRI<sup>c</sup>* region in ADR1 function. An internal in-frame deletion between amino acids 220 and 262 of ADR1, the entire *ADRI<sup>c</sup>* region, was constructed and integrated as a single copy into the genome to address this question. The resulting *ADRI-220/262* allele was found to result in an *ADH2* phenotype that was very similar to that obtained with the *ADRI<sup>c</sup>* mutations (Table 3). Under glucose growth conditions, *ADH2* expression in the strain carrying the deletion was observed to be 30-fold higher than that found in a wild-type strain. We also observed that this effect of *ADRI-220/262* on *ADH2* expression was not a result of a corresponding increase in the level of ADR1-220/262 protein in the cell relative to the level of ADR1 protein as quantitated by immunoprecipitation of ADR1-220/262 (data not shown) (9). The observation that the deletion of the residues between 220 to 262 resulted in elevated *ADH2* expression suggests, therefore, that the *ADRI<sup>c</sup>* region plays a negative role in ADR1 function. These results support the model that

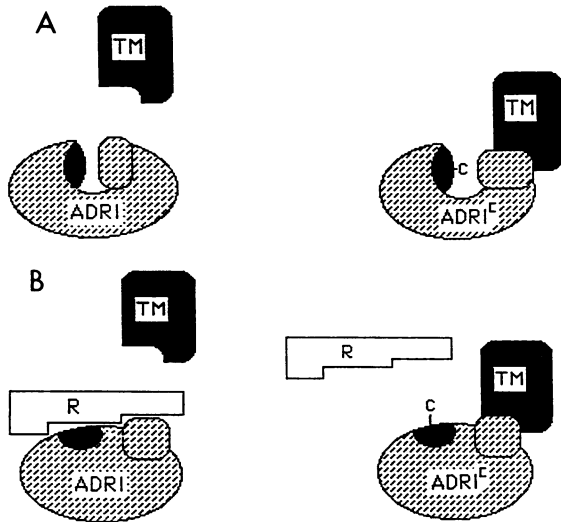


FIG. 2. Models for the role of the *ADRI<sup>c</sup>* region. (A) The 227 to 239 region forms an intrasteric interaction and binds up an *ADRI* activation domain. An *ADRI<sup>c</sup>* alteration allows the activation domain to make contact with the transcriptional machinery (TM). (B) A repressor (R) binds the 227 to 239 region and blocks *ADRI* activation of transcription. An *ADRI<sup>c</sup>* alteration prevents the repressor from binding and allows *ADRI* to contact the transcriptional machinery. The *ADRI<sup>c</sup>* region is darkened, and the putative *ADRI* activation domain is encircled.

the *ADRI<sup>c</sup>* mutations enhance *ADRI* activity by altering the structure of the region and therefore its contact with another protein or another region of *ADRI*.

## DISCUSSION

Our previous studies indicated that *ADRI<sup>c</sup>* mutations reduce or eliminate cAPK phosphorylation of Ser-230 *in vitro* (6). Unregulated cAPK was subsequently shown to inhibit *ADH2* expression (6) in an *ADRI*-dependent manner (10). *ADRI<sup>c</sup>* mutations displayed reduced sensitivity to unregulated cAPK activity (6). These results suggested that cAPK inactivates *ADH2* expression by inhibiting *ADRI* function through phosphorylation of Ser-230.

The data we present here, however, do not support the model that the *ADRI<sup>c</sup>* mutations enhance *ADRI* function by solely affecting cAPK phosphorylation of Ser-230. We observed that several newly characterized *ADRI<sup>c</sup>* mutations did not affect the ability of cAPK to phosphorylate Ser-230 on synthetic peptides modeled on the *ADRI<sup>c</sup>* region. Most importantly, we found that strains lacking cAPK activity did not evince an *ADRI<sup>c</sup>* phenotype. The *ADRI<sup>c</sup>* mutations, therefore, must be activating *ADRI* by a mechanism that is independent of interference with cAPK phosphorylation of Ser-230. Moreover, it was observed that when *ADRI* residue 230 could not be phosphorylated, the lack of cAPK caused an increase in *ADRI* function. This result supports the observation that the cAPK regulation of *ADH2* does not occur directly through phosphorylation of Ser-230. Thus, the previous model suggesting that Ser-230 of *ADRI* was the site of this inactivation is probably incorrect.

Our previous papers (6, 10) and the present data (Tables 5 and 6) clearly indicate that cAPK inhibits *ADH2* expression. That this inhibition is physiologically relevant and is not an artifact of unregulated cAPK activity is evidenced by the observation that lack of cAPK activity augmented *ADH2*

expression in an *ADRI<sup>-</sup>* background (Table 6). We have also established that *ADRI* function is required for the effect of unregulated cAPK activity on derepressed *ADH2* expression (10). These results imply that cAPK inhibits *ADH2* by phosphorylating either *ADRI* directly or another protein required for *ADRI* activity. The identity of the other putative protein, however, remains unknown. The two other protein kinases (SNF1 and SCH9) which control *ADH2* expression are not intermediaries for the cAPK effect, since both proteins have been shown to act independently of cAPK and *ADRI* in controlling *ADH2* expression (10). Our previous observation that *ADRI<sup>c</sup>* alleles were less sensitive to unregulated cAPK activity than the wild-type *ADRI* gene led us to postulate that Ser-230 was the site of cAPK control *in vivo* (6). This clearly cannot be the case, as described above. *ADRI<sup>c</sup>* proteins may instead be able to partially bypass the effects of unregulated cAPK on *ADH2* expression by altering *ADRI* structure and hence responsiveness at other sites. Alternatively, the mutated proteins may reduce cAPK effects by affecting the functions of proteins required for *ADRI* action. In either case, the mechanism by which *ADRI<sup>c</sup>* mutations activate *ADRI* is distinct from that exercised by cAPK in controlling *ADH2* expression.

In addition, because a lack of cAPK activity did not release *ADH2* expression from glucose repression in an *ADRI* background, cAPK does not appear responsible for the glucose-to-ethanol transition in controlling *ADH2* expression. Other factors must first bring about these effects. This suggestion is consistent with the observation that *ADRI* must be in an altered structural state (*ADRI<sup>c</sup>* or the derepressed form) in order for cAPK to affect *ADH2* expression. cAPK appears, therefore, to control the level of *ADRI* activity but not necessarily the carbon source regulation of *ADRI*.

How do *ADRI<sup>c</sup>* mutations enhance *ADRI* function if they do not affect cAPK phosphorylation of Ser-230? *ADRI<sup>c</sup>* alterations could be affecting the ability of a protein kinase other than cAPK to phosphorylate Ser-230. However, the phosphorylation state of Ser-230 may be only one factor that contributes to the integrity and function of the *ADRI<sup>c</sup>* region, since we observed that several *ADRI<sup>c</sup>* mutations resulted in higher *ADH2* expression than *ADRI-13<sup>c</sup>*, which cannot be phosphorylated at Ser-230 (Table 3). Because we demonstrated that deletion of the *ADRI<sup>c</sup>* region (removal of amino acids 220 to 262) conferred an *ADRI<sup>c</sup>* phenotype, the region where these mutations are located represents an inhibitory domain of *ADRI*. This domain may bind a repressor (see Fig. 2B) that maintains *ADRI* in an inactive state through inhibition of its ability to activate transcription. *ADRI<sup>c</sup>* alterations would prevent the repressor from binding to *ADRI*. Alternatively, the *ADRI<sup>c</sup>* domain may block the ability of another segment of *ADRI* to activate transcription through an intrasteric interaction (Fig. 2A). We favor the latter model for two reasons. There is no genetic evidence indicating the presence of a repressor that binds *ADRI*. Also, overproduction in *S. cerevisiae* of the protein region encompassing the *ADRI<sup>c</sup>* region was not observed to affect *ADH2* expression, suggesting that there is no titratable repressor that binds to *ADRI* (unpublished observations). In either case, the fact that all 21 *ADRI<sup>c</sup>* mutations were observed to occur in a 13-amino-acid stretch between residues 227 and 239 indicates that this region is structurally important to maintaining *ADRI* in an inactive state.

Many other eucaryotic transcriptional activators contain regions which serve to inhibit their ability to turn on transcription. These include the yeast activators GAL4 (23, 28)

and PUT3 (29), as well as the mammalian factors CREB (18), C/EBP (34), and c-Myb (27). Restricting an activator's ability to stimulate transcription by the binding of a mask appears, therefore, to be a common mechanism. The masks may be other proteins that bind and repress function, as in the GAL80 inhibition of GAL4 (23, 28), or they may be internal regions that block the activation function through an intrasteric interaction. This phenomenon resembles that of pseudosubstrate regulation, which was first clearly outlined for protein kinases (for a review, see reference 25). The pseudosubstrate mimics the substrate and binds at the active site, preventing the protein kinase from phosphorylating its normal target substrates. The pseudosubstrate may be located either on a different protein, as is the case with cAPK (33), or on the same protein, as found for myosin light chain protein kinase and protein kinase C (21, 33). For the transcriptional activators, it is not clear whether the inhibitory domains resemble the factors to which the activation domains bind or whether the inhibitory regions indirectly block the activation regions.

It should also be noted that lack of cAPK activity in an *ADRI*<sup>-</sup> background caused an 8- to 15-fold increase in *ADH2* expression under glucose-repressed conditions but resulted in only a twofold elevation under ethanol growth conditions. This discrepancy is most likely due to the fact that under glucose conditions *ADH2* expression is strictly *ADRI*-dependent, whereas under derepressed conditions other factors are clearly limiting (9). Enhancing the ability of *ADRI* to activate results in large effects on *ADH2* expression under repressed conditions but produces much smaller effects under derepressed conditions (2, 9).

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