

Snf1 Controls the Activity of Adr1 Through Dephosphorylation of Ser230

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ABSTRACT

The transcription factors Adr1 and Cat8 act in concert to regulate the expression of numerous yeast genes after the diauxic shift. Their activities are regulated by Snf1, the yeast homolog of the AMP-activated protein kinase of higher eukaryotes. Cat8 is regulated directly by Snf1, but how Snf1 regulates Adr1 is unknown. Mutations in Adr1 that alleviate glucose repression are clustered between amino acids 227 and 239. This region contains a consensus sequence for protein kinase A, RRAS₂₃₀F, and Ser230 is phosphorylated *in vitro* by both protein kinase A and Ca⁺⁺ calmodulin-dependent protein kinase. Using an antiphosphopeptide antibody, we found that the level of Adr1 phosphorylated on Ser230 was highest in glucose-grown cells and decreased in a Snf1-dependent manner when glucose was depleted. A nonphosphorylatable Ser230Ala mutant was no longer Snf1 dependent for activation of Adr1-dependent genes and could suppress Cat8 dependence at genes coregulated by Adr1 and Cat8. Contrary to expectation, neither protein kinase A (PKA) nor Ca⁺⁺ calmodulin-dependent protein kinase appeared to have an important role in Ser230 phosphorylation *in vivo*, and a screen of 102 viable kinase deletion strains failed to identify a candidate kinase. We conclude that either Ser230 is phosphorylated by multiple protein kinases or its kinase is encoded by an essential gene. Using the Ser230Ala mutant, we explain a long-standing observation of synergy between Adr1 constitutive mutants and Snf1 activation and conclude that dephosphorylation of Ser230 via a Snf1-dependent pathway appears to be a major component of Adr1 regulation.

TRANSSCRIPTION factors activate gene expression by recruiting coactivators to a promoter to create a pre-initiation complex (PIC) containing RNA polII and general factors (FEATHERSTONE 2002; FRY and PETERSON 2002). Signal transduction pathways often regulate this process by acetylation or phosphorylation of transcription factors to influence their ability to recruit coactivators (KWOK *et al.* 1994; PAPOUTSPOULOU and JANKNECHT 2000; ZHONG *et al.* 2002; GOEL and JANKNECHT 2003). Adr1 is a yeast transcription factor that acts in concert with Cat8 and Oaf1/Pip2 to activate numerous genes that are expressed after glucose depletion, allowing cells to use nonfermentable carbon sources such as ethanol, glycerol, and fatty acids (SCHULLER 2003; YOUNG *et al.* 2003; SANTANGELO 2006). The signal transduction pathway that activates Adr1 and Cat8 is mediated by Snf1 (RAHNER *et al.* 1999; SCHULLER 2003; CHARBON *et al.* 2004; SANTANGELO 2006), the yeast homolog of AMP-activated protein kinase that is widely regarded as the “energy sensor of the cell” (HARDIE *et al.* 1998). Snf1 is activated by phosphorylation on Thr210 by three upstream kinases (HONG *et al.* 2003). Snf1-Thr210 is dephosphorylated by the PP1-type protein phosphatase

Glc7, and regulation of this event appears to be through the targeting factor Reg1 (SANZ *et al.* 2000; RUBENSTEIN *et al.* 2008). As expected, removing the negative regulatory mechanism by deleting *REG1* causes constitutive activation of Snf1, as measured by expression in glucose of Snf1-dependent genes that are normally glucose repressed (DOMBEK *et al.* 1993; TU and CARLSON 1995; MCCARTNEY and SCHMIDT 2001; ORLOVA *et al.* 2008). Control of Snf1 must include additional mechanisms beyond simple phosphorylation and Glc7-Reg1-dependent dephosphorylation, however, because in a *reg1Δ* strain, additional phosphorylation of Snf1 and further activation of some Snf1-dependent genes is achieved by glucose depletion (DOMBEK *et al.* 1993; TACHIBANA *et al.* 2007; ORLOVA *et al.* 2008). Interaction with regulatory proteins or changes in protein structure have been suggested as additional levels of Snf1 control (RUBENSTEIN *et al.* 2008).

Snf1 is necessary for the activity of both Adr1 and Cat8, enhancing transcription of *CAT8* through inactivation of the repressor Mig1, as well as activating Cat8 directly by phosphorylation (HEDGES *et al.* 1995; RAHNER *et al.* 1996, 1999; CHARBON *et al.* 2004; reviewed in SCHULLER 2003). Snf1 is required for Adr1 binding (YOUNG *et al.* 2002) and recruitment of coactivators to Adr1-dependent promoters (BIDDICK *et al.* 2008). Promoter binding by Adr1 is regulated in part by phosphorylation of its DNA-binding domain (KACHEROVSKY *et al.* 2008) and by acetylation of promoter nucleosomes (VERDONE *et al.*

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2002; TACHIBANA *et al.* 2007), but it is not known whether Snf1 activates Adr1 directly or indirectly or has another role at Adr1-dependent promoters, such as nucleosome modification (LO *et al.* 2001).

Selection of mutants that allowed *ADH2* expression to escape glucose repression identified rare semidominant *ADRI^c* alleles (*ADRI*-constitutive) (CIRIACY 1979; DENIS *et al.* 1991). *ADRI^c* alleles allow *ADH2* expression to occur in the presence of glucose and enhance derepressed transcription. Surprisingly, the level of Adr1^c protein is much lower than the level of wild-type Adr1 (TAYLOR and YOUNG 1990; DOMBEK and YOUNG 1997), suggesting that its high transcriptional potency might be associated with rapid turnover as has been observed for some other activators (TANSEY 2001). *ADRI^c* shows strong synergism with deletion of the Snf1 regulatory factor *REG1*. In combination with Adr1^c, partial activation of Snf1 by deleting *REG1* allows much higher levels of *ADH2*-repressed expression than are observed with either mutation acting alone (DOMBEK *et al.* 1993). Adr1^c and *reg1Δ* are also synergistic in activating a poised but inactive PIC (TACHIBANA *et al.* 2007). Although this suggests that Adr1^c and activation of Snf1 by *reg1Δ* act through different pathways to cause constitutive expression of Snf1-dependent genes, the precise mechanism of the synergism is unknown.

Cloning and molecular analysis of *ADRI^c* alleles showed mutations between amino acids 227 and 239, a region that contains a consensus sequence for a cyclic AMP-dependent PKA, Arg-Arg-Ala-Ser-Phe (DENIS and GALLO 1986; DENIS *et al.* 1992). No other mutations in the Adr1 ORF causing constitutive *ADH2* expression were isolated, suggesting that the 227–239 region plays a unique role in regulating Adr1 activity. The *ADRI^c* mutations suggest that Adr1 might be post-translationally inhibited by phosphorylation of Ser230 in the PKA consensus sequence (DENIS and GALLO 1986; CHERRY *et al.* 1989). Adr1 appears to be phosphorylated at Ser230 *in vivo* because subtle alterations in its SDS-PAGE electrophoretic mobility and partial sensitivity to phosphatase treatment are observed (VALLARI *et al.* 1992; DOMBEK and YOUNG 1997). Recent studies of the yeast phosphorylome also suggested Adr1 phosphorylation, although the reported site was Ser232, not Ser230 (CHI *et al.* 2007).

In support of phosphorylation of Adr1 by PKA, both PKA and calmodulin-dependent protein kinase (CMK) efficiently phosphorylate Ser230 *in vitro* (DENIS *et al.* 1991; HOOK *et al.* 1999). Hyperactivation of PKA by deletion of its *BCY1*-encoded regulatory subunit causes loss of *ADH2* expression (CHERRY *et al.* 1989), suggesting that unregulated PKA activity inhibits Adr1 by constitutively phosphorylating Ser230. However, replacing Ser230 with phosphomimetic Asp gave the same constitutively active phenotype as other alterations in the region, suggesting that either Asp does not mimic phosphorylated Ser or phosphorylation of Ser230 is not

the only determinant of the *ADRI^c* phenotype (DENIS *et al.* 1992; DOMBEK and YOUNG 1997). In addition, several of the Adr1^c mutations appear to refute PKA as a candidate for the Ser230 kinase *in vivo*. The Ala229Pro Adr1^c mutant shows greatly increased affinity for PKA binding in a peptide phosphorylation assay, which would predict greater phosphorylation and lower Adr1 activity. However, the Ala229Pro mutant is one of the most constitutive of the Adr1^c mutants (DENIS *et al.* 1992). Therefore, it is not known if phosphorylation of Ser230 affects the activity of Adr1 and what kinase is responsible for the phosphorylation *in vivo* (DENIS *et al.* 1992; COOK *et al.* 1994; DOMBEK and YOUNG 1997).

How Adr1^c achieves its high transcriptional potency is unknown (DENIS *et al.* 1992; COOK *et al.* 1994; DOMBEK and YOUNG 1997). *ADRI^c* alleles map far from the DNA-binding domain and Adr1-Ser230A does not have enhanced *in vitro* DNA-binding activity (TAYLOR and YOUNG 1990). After characterizing a series of mutants in amino acids 227–239, as well as internal deletions in this region of Adr1, COOK *et al.* (1994) suggested that this region either binds a repressive factor that cannot interact with the mutants or interacts with and blocks the activation domain. Some evidence has suggested that these Adr1^c mutants suppress the requirement for Snf1 (CIRIACY 1979; DENIS 1984), which would imply that it is not affected by Snf1, but this is contradictory to the enhanced effect on *ADH2* expression seen when Adr1^c is combined with the Snf1-activating *reg1Δ* mutation. Thus, the questions of how Snf1 affects the activity of Adr1 and whether it interacts with the Adr1^c allele are unresolved.

We used an antibody against a Ser230-phosphorylated peptide of Adr1 amino acids 217–234 to investigate the conditions under which Ser230 is phosphorylated (Adr1-pSer230) and the effect of Snf1 on this modification. The antibody was used to screen for a specific Adr1-Ser230 kinase. Phosphorylation was not increased by enhanced PKA activity, nor decreased by loss of PKA or CMK. These and other data suggest that Ser230 may be redundantly phosphorylated *in vivo* by multiple kinases or phosphorylated by an essential kinase. Using the nonphosphorylatable Adr1^c, we probed the relationship between Adr1-pSer230, Snf1, and the coregulator Cat8, with the results supporting an earlier model for the function of the Ser230-containing domain.

MATERIALS AND METHODS

Yeast strains, plasmids, culture conditions, and enzyme assays: The strains used in the study are listed in Table 1. Multiple deletion mutants of *TPK* and *CMK* genes were constructed by standard genetic techniques. Epitope tagging, gene deletion, and marker swapping were according to GULDENER *et al.* (1996), CROSS (1997), and KNOP *et al.* (1999), respectively. Deletions and epitope tagging were confirmed by PCR and, for marker swapping, by Western blots. Yeast strains

TABLE 1
S. cerevisiae strains

Strain	Genotype	Source
TYY201 (aka W303-1A)	<i>MATa ade2 can1-100 his3-11,15 leu2-13,112 trp1-1 ura3-1</i>	Yeast stock center
VBY20	<i>MATα adh3 ura3 his3 leu2::(pRS315-ADR1)X3 ADH2::YIp ADH2/lacZ(TRP1)ADH2::YIpADH2/GFP (URA3)</i>	VORONKOVA <i>et al.</i> (2006)
NKY85	VBY20 <i>ADR1:HA::kanMX</i>	This study
NKY87	NK85 <i>reg1Δ::natMX</i>	This study
NKY91	NK85 <i>snf1Δ::natMX</i>	This study
TYY204	W303-1A <i>adr1Δ1::LEU2 ADH2::YIpADH2/lacZ::TRP1</i>	YOUNG <i>et al.</i> (2003)
TYY497	W303-1A <i>adr1Δ1::LEU2 ADH2::YIpADH2/lacZ::trp1::HIS3</i>	YOUNG <i>et al.</i> (2008)
TYY498	W303-1A <i>snf1Δ::URA3 adr1Δ1::LEU2 ADH2::YIpADH2/lacZ::trp1::HIS3</i>	YOUNG <i>et al.</i> (2008)
CKY13	W303-1A <i>adr1Δ::kanMX</i>	This study
CKY26	W303-1A <i>adr1Δ::natMX snf1Δ::kanMX</i>	This study
CKY5	W303-1A <i>adr1Δ::kanMX reg1Δ::natMX</i>	This study
CKY23	W303-1A <i>adr1Δ::natMX cat8Δ::kanMX</i>	This study
SRY60	W303-1A <i>reg1Δ::kanMX cat8Δ::natMX adr1Δ::HYG</i>	This study
NKY108	NKY85 <i>cmk1Δ::hphmx cmk2Δ::natMX cmk3Δ::natMX</i>	This study
SGP406	<i>MATa his3 leu2-3,112 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 trp1 ura3-52 yak1::LEU2</i>	GARRETT and BROACH (1989)
NKY111	NKY85 <i>bcy1Δ::natMX</i>	This study
CMY323	<i>MATa ade2-101° can1 his3Δ200 lys2-801 trp1Δ1 ura3-52</i>	Carl Mann
CMY328	<i>MATa ade2-101° adr1Δ2::URA3 can1 his3Δ200 lys2-801atrp1Δ1 ura3-52</i>	DOMBEK and YOUNG (1997)
CMY468	<i>MATa ade2-101° his3Δ leu2Δ1 lys2-801 tpk1::URA3 tpk2::HIS3tpk3ts-1 trp1Δ1 ura3-52</i>	Carl Mann
SGP400	<i>MATa his3 leu2-3,112 trp1 ura3-52 yak1::LEU2</i>	GARRETT and BROACH (1989)

were grown in complete or synthetic media as described in SHERMAN (1991). Repressing medium contained 5% glucose; derepressing medium contained 0.05% glucose with or without 3% glycerol or 2% ethanol. The plasmids used were described previously (YU *et al.* 1989; DOMBEK and YOUNG 1997) and modified in some cases by introducing an epitope tag at the C terminus of Adr1 as described below. For gene expression studies, *CEN-TRP1* plasmids expressing wild-type *ADR1* (pKD16) or the S230A (pKD14), R228K (pKD27), or Δ3 (a deletion that removes Adr1 amino acids 226–233; pKD26) alleles of *ADR1* from its native promoter were used. To facilitate detection of Adr1-S230 phosphorylation by Western blotting, 2μ plasmids expressing wild-type *ADR1* (pKD17-HA::kanMX (*TRP1*)) and the *ADR1*-S230A allele (pKD20-HA::kanMX (*TRP1*)) from the *ADH1* promoter and tagged with an HA epitope were employed. Alternatively, strains with four additional copies of *ADR1* integrated at the *leu2* locus were used (SLOAN *et al.* 1999). *ADR1* was epitope tagged with HA::kanMX in this strain. The Adr1-dependent reporter was a UAS1-*lacZ* plasmid, pHDY10, containing 10 Adr1-binding sites (YU *et al.* 1989). β-Galactosidase assays were performed as described in GUARENTE (1983). ADH enzyme activity was analyzed by separating proteins on nondenaturing polyacrylamide gels and visualized by in-gel chromogenic staining as described (DOMBEK and YOUNG 1997).

Real-time quantitative PCR: For expression analysis, RNA was isolated by hot phenol extraction (COLLART and OLIVEIRO 1993) and converted to cDNA with a SuperScript III kit (Invitrogen) according to the manufacturer's directions. cDNA was quantified by real-time quantitative PCR (RT-qPCR) (TACHIBANA *et al.* 2007) with an MJ Research Chromo4 system, using ABI or Quantace SYBR Master Mix, according to the manufacturer's instructions. Primer sequences are available on request.

Immunoblotting: Whole-cell extracts were analyzed on 3–8% or 6% polyacrylamide gels (NuPage system, Invitrogen) and transferred to PVDF membranes after electrophoresis. Western blots were probed with α-pSer230 followed by a rabbit-specific Licor secondary Ab (λ680). A second identical blot was probed with a monoclonal α-HA antibody and a mouse-specific Licor secondary antibody (λ800). Probing a single blot with both antibodies resulted in bleed-through from the more abundant HA-epitope-derived signal so either two blots were done or a single blot was probed sequentially—first, with α-pSer230 and an appropriate Licor secondary antibody and, second, with the α-HA antibody and an appropriate Licor secondary antibody. Cell extracts were prepared as described previously (DOMBEK and YOUNG 1997) or by a boiling procedure with the addition of agitation with glass beads for 30 sec before the boiling step (Invitrogen) (HAHN 2008). An Odyssey Infrared imaging system (Licor Biosciences) was used for quantitation. α-HA (Y-11, Santa Cruz Biochemicals), α-Adr1 (DOMBEK *et al.* 1993), or antibodies against an Adr1-derived synthetic peptide were used at 1:1000 dilution. The peptides, representing Adr1 amino acids 217–234, VKRKYLKLLTRRA (pS)FSAQ, and its nonphosphorylated version were synthesized and used to generate and affinity purify the α-pSer230 antibody by Bethyl Laboratories (Montgomery, TX). Secondary IR-dye-conjugated antibodies used at 1:1000–1:3000 were goat α-mouse Alexa 680 (Molecular Probes) or IRdye800-conjugated α-rabbit IgG (Rockland Immunochemicals).

RESULTS

Adr1-Ser230 phosphorylation *in vivo* is glucose dependent and dephosphorylation is Snf1 dependent: Antibodies against a phosphorylated peptide represent-

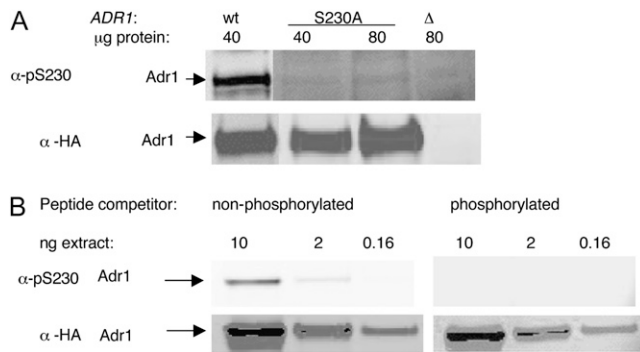


FIGURE 1.—Antiphosphopeptide antibody recognizes Adr1 phosphorylated on Ser230. (A) α -pSer230 recognizes Adr1 in whole-cell extracts. Wild type (wt) and S230A refer to TYY497 (*adr1 Δ 1*) with pKD17-HA and pKD20-HA, expressing HA-epitope-tagged versions of wild-type *ADR1* or *ADR1-Ser230*, respectively; “ Δ ” is the strain TYY497 with a vector lacking *ADR1*. The amount of protein loaded on the gel is shown above each lane. The cells were grown in *trp*[−] synthetic medium in repressing conditions (5% glucose). (B) Peptide competition. Ten ng/ml of nonphosphorylated or Ser230-phosphorylated peptide (MATERIALS AND METHODS) representing Adr1 amino acids 217–234 was present during Western blotting with the primary antibody. Cell extracts were made from strain TYY497 carrying pKD17-HA (wt multicopy *ADR1*) grown in repressing conditions in *trp*[−] synthetic medium. The amount of protein loaded on the gel is shown above each lane. A very low amount of cell extract (nanograms) was used to allow effective competition with the peptide.

ing Adr1 amino acids 217–234 (VKRKYLKKLTRRA (pS)FSAQ) were generated to assess the level of Adr1 phosphorylated on Ser230 *in vivo* (designated Adr1-pSer230). ELISA assays indicated that the antiphosphopeptide antibody (α -pSer230) recognized the phosphorylated peptide 5000 \times better than the non-phosphorylated peptide (data not shown). Figure 1A shows that α -pSer230 recognized a protein the size of Adr1 in a strain expressing Adr1-HA from the strong *ADH1* promoter on a multicopy plasmid, but not in a strain lacking Adr1. Adr1 containing a Ser230Ala mutation (hereafter referred to as Adr1^c unless other *ADR1*^c alleles are specified) was recognized poorly by α -pSer230, demonstrating the specificity of the antibody for Adr1 with phosphorylated Ser230. Competition with the phosphorylated and nonphosphorylated peptides demonstrated the specificity of α -pSer230 (Figure 1B). These data confirmed that α -pSer230 recognized Adr1-pSer230 and cross-reacted weakly, if at all, with Adr1 lacking phosphate on Ser230.

A strain containing multiple integrated copies of *ADR1* was employed to facilitate quantitation of Adr1-pSer230 in different growth conditions. At least one copy of Adr1 in this strain was tagged with an HA epitope to provide a measure of the total amount of Adr1 in the extract. Adr1 levels in the multicopy strain grown in repressing conditions are similar to Adr1 levels in wild-type cells grown in derepressing conditions, and

ADH2 regulation appears unperturbed by the modest increase in Adr1 levels (SLOAN *et al.* 1999; VORONKOVA *et al.* 2006).

Western blotting for both HA and Adr1-pSer230 revealed that the amount of pSer230 relative to total Adr1 was highest in glucose-grown cells and decreased \sim 10-fold when cells were grown in low glucose plus ethanol (Figure 2A, wt lanes). The decrease in pSer230 in the absence of glucose was not due to the elevated Adr1 level in the multicopy strain because a decrease was also observed in a strain with a single copy of *ADR1* (Figure 2B). Although the low level of Adr1-pSer230 in the single-copy strain precluded quantification of the change in phosphorylation, Adr1 increased at least 10-fold after 22 hr of growth in the absence of glucose, and the level of pSer230 decreased to an undetectable level after 2 hr.

A strain lacking *SNF1* is unable to activate *ADH2* expression presumably due to inactive Adr1. Consistent with the hypothesis that phosphorylation of Ser230 inactivates Adr1, in a *snf1 Δ* strain, the amount of Adr1-pSer230 relative to total Adr1 appeared to be elevated in the presence of glucose and did not decrease significantly after its removal (Figure 2A, *snf1 Δ* lanes). Since Snf1 is a kinase, any regulation of phosphorylated Ser230 must be indirect, for example, through control of another kinase or a phosphatase. Surprisingly, although dephosphorylation of Ser230 appeared to be Snf1 dependent, deleting *REG1* or inactivating *GLC7* with a temperature-sensitive mutation to activate Snf1 in repressing conditions did not affect the relative level of Adr1-pSer230 in either repressing or derepressing conditions (Figure 2A, *reg1 Δ* ; unpublished data for *Glc7-127*). The experiment was repeated multiple times with similar results. Snf1 control of Adr1-pSer230 apparently requires additional levels of Snf1 regulation, such as control over access to the Snf1 T210 phosphorylation site, that work with dephosphorylation by Reg1-Glc7 to mediate Snf1 activity (ORLOVA *et al.* 2008; RUBENSTEIN *et al.* 2008). Alternatively, Ser230 dephosphorylation in glucose may be prevented by a mechanism that is independent of Reg1-Glc7. In summary, inactivation of Snf1 prevented the decrease in Ser230 phosphorylation that normally accompanies derepression and activation of Adr1-dependent genes, which is consistent with phosphorylation playing a negative role in regulating the activity of Adr1.

The stoichiometry of phosphorylation on Ser230 was assessed by immunoprecipitation of Adr1 in whole-cell extracts derived from the multicopy *ADR1* strain grown in repressing conditions. Adr1 was immunoprecipitated with α -Adr1 and α -pSer230 antisera, and the precipitated and supernatant fractions were analyzed by SDS-PAGE. Western blotting using α -Adr1 antisera, which recognizes both phosphorylated and nonphosphorylated Adr1, demonstrated that α - and α -pSer230 antisera immunoprecipitated 65% and 45%, respectively, of the total Adr1 (supporting information, Figure S1). De-

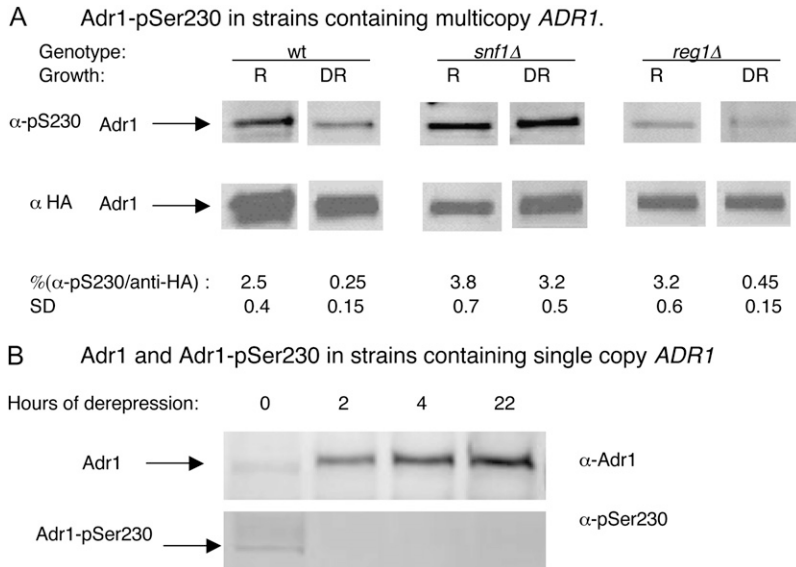


FIGURE 2.—Phosphorylation of Adr1-pSer230 is glucose and Snf1 regulated. Preparation of cell extracts, electrophoresis, and Western blotting were carried out as described in MATERIALS AND METHODS. (A) Multicopy *ADR1* strains. Wild type (wt) is NKY85; *snf1Δ* is NKY91, and *reg1Δ* is NKY87; R, repressing growth conditions, 5% glucose; derepression (DR) was for 6 hr in 0.05% glucose–2% ethanol. Cell extract (25 μg) was analyzed. The signal intensity was measured as described in MATERIALS AND METHODS for several biological replicates. The values shown are the means ± one standard deviation (SD). (B) A single copy *ADR1* strain (TYY201) was analyzed in a similar manner using 100 μg of protein extracted from repressed cells (0 hr derepression) and from cells derepressed for 2, 4, and 22 hr in 0.05% glucose–2% ethanol.

phosphorylation of pSer230 during immunoprecipitation may contribute to the lower level of Adr1 immunoprecipitated by the α-pSer230 antisera compared to the α-Adr1 antisera, or there may be a fraction of Adr1 that is not phosphorylated on Ser230 even in repressing growth conditions. The high level of Adr1 precipitated by the α-pSer230 antisera indicates that the majority of Adr1 is phosphorylated *in vivo* in repressing conditions.

Adr1^c activation is Snf1 independent and synergism with *reg1Δ* is from Snf1 activation of Cat8: Since the decrease of Adr1-Ser230 phosphorylation did not occur in a *snf1Δ* strain, we hypothesized that, if this decrease were important for the activity of Adr1, Adr1^c, which has the phosphorylatable serine mutated to alanine, would be insensitive to *snf1Δ*. As predicted, both *ADH2/lacZ* reporter assays and in-gel assays of endogenous ADHII activity showed that Adr1^c was highly efficient at activating *ADH2*, even in the absence of Snf1. *ADH2* derepression by Adr1^c was reduced only fourfold in the *snf1* mutant, compared to an almost complete lack of expression with wild-type Adr1 (Figure S2). Snf1 independence of Adr1^c was also seen when mRNA from *ADH2* was measured directly by RT–qPCR. With this assay, we found that other Adr1-dependent genes, including *POX1* and *SPS19*, were also activated in a Snf1-independent manner by Adr1^c. Adr1^c did not change the expression of *FBP1*, which is not Adr1 dependent (Figure 3A). Other Adr1^c variants of the Ser230 region were used to show that the Snf1 independence was a general effect, because two other alleles, Adr1-Δ3 [Δ(226-223)] and Adr1-R228K, also activated the Adr1-dependent genes without Snf1. The *CAT8*-dependent *FBP1* gene was not derepressed in *snf1Δ* strains by Adr1^c alleles (Figure 3B). *ADR1^c* could not overcome the Snf1 requirement for growth on the nonfermentable carbon sources ethanol, glycerol, lactate, or acetate, however (data not shown). This is not surprising because Cat8

is essential for growth on these carbon sources and expression of Cat8-dependent genes such as *FBP1* were unaffected by Adr1^c. In addition, there are other Snf1-dependent transcription factors such as Mig1 that are involved in gene expression for these metabolic pathways.

The lack of Snf1 control over Adr1^c alleles raised a question about earlier results showing synergism between Adr1^c and *reg1Δ* (DOMBEK *et al.* 1993; TACHIBANA *et al.* 2007), in which Adr1^c-containing strains show additional activation of *ADH2* when Snf1 is activated in glucose by *reg1Δ*. Adr1^c was Snf1 independent, so it should not be influenced by *reg1Δ*. A clue to the mechanism of the synergism came from the high Snf1 dependence seen for the Cat8-dependent *FBP1* gene (Figure 3, A and B) and the lack of synergism seen at Cat8-independent genes such as *POX1* (data not shown). We hypothesized that the additional activation of *ADH2*, seen when *REG1* was deleted in an Adr1^c-containing strain, is from Snf1 activation of Cat8. If this hypothesis is correct, reducing the Cat8 effect should reduce the synergism. We measured *ADH2* activation independently of other activators using a reporter that lacked binding sites for Cat8 and had only the Adr1-binding site (UAS1) of the *ADH2* promoter (YU *et al.* 1989). Snf1 dependence and synergism with *reg1Δ* was determined in strains carrying the reporter and either wild-type Adr1 or Adr1^c. The activity of the reporter in the *REG1* strain with wild-type Adr1 was very low in repressing conditions and increased 260-fold in derepressing conditions (Table 2). The Adr1^c allele showed the expected constitutive activity and hyperactivation. In the *reg1Δ* mutant, wild-type Adr1 showed constitutive β-galactosidase activity to ~10% of the 8-hr derepressed value in the *REG1* strain. The same degree of constitutive activity was seen when Adr1^c was the activator in the *reg1Δ* strain, instead of the synergistic effects seen previously in assays that measured expression from the entire *ADH2* promoter

A Adr1^c suppresses *snf1Δ* defects in ADR1-dependent gene expression

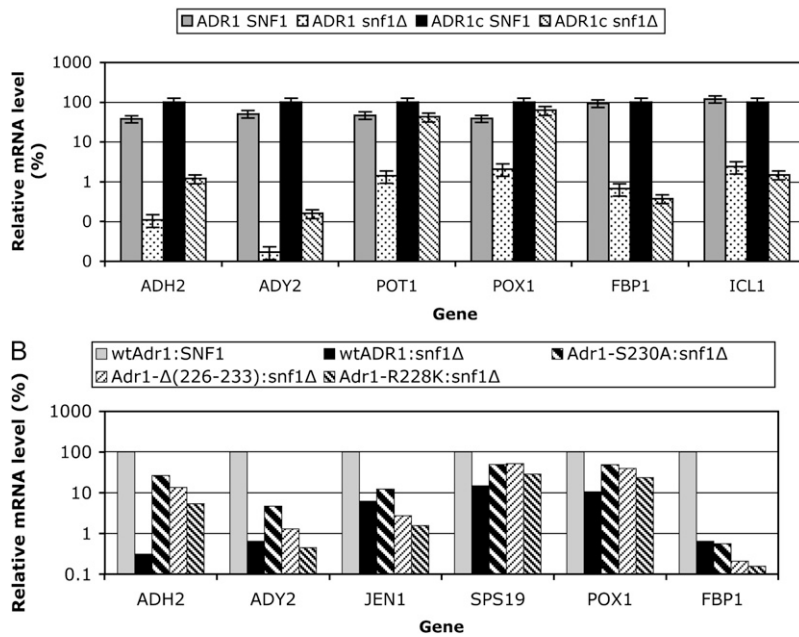


FIGURE 3.—Snf1 dependence of Adr1^c-dependent gene expression. (A) Derepressed transcript levels in *SNF1* or *snf1Δ*, with wild-type (wt) *ADR1* or *ADR1^c* (*ADR1-S230A*). Analysis of mRNA isolated from cells derepressed in 0.05% glucose–3% glycerol for 4 hr. RT–qPCR was performed and analyzed as described in MATERIALS AND METHODS. Strains were TYY497 (*adr1ΔSNF1*) and TYY498 (*adr1Δsnf1Δ*) carrying either wild-type *ADR1* (pKD16) or *ADR1-S230A* (pKD14). The mRNA values were normalized to *ACT1* mRNA and are expressed as a percentage of mRNA in the wild-type *SNF1* strain with wild-type Adr1 as the activator. The values represent the mean ± a standard deviation of two biological replicates assayed in duplicate. (B) Relative transcript levels in *snf1Δ* with various Adr1^c alleles. Transcript levels were quantified and displayed as in A. The strains were CKY13 (*adr1Δ*) and CKY26 (*adr1Δ snf1Δ*). The plasmids were as in section A and additionally pKD26 [Adr1-Δ(226–233)] and pKD27 (Adr1-R228K).

(DOMBEK *et al.* 1993; TACHIBANA *et al.* 2007). In the *reg1Δ* strain, both the wild-type and Adr1^c alleles showed a slight increase in constitutive activity over the *REG1* strain, possibly due to the 2- to 3-fold elevated *ADR1* transcription that is observed in a *reg1Δ* mutant (DOMBEK *et al.* 1993).

We additionally tested the reliance of Adr1^c and *reg1Δ* synergism on Cat8 by deleting *CAT8* in an *adr1Δ reg1Δ* mutant strain and by introducing wild-type Adr1 or Adr1^c on plasmids. *ADH2* expression was measured by isolating mRNA and performing RT–qPCR. The results indicated that Cat8 was required for the high, glucose-resistant *ADH2* expression in the *reg1Δ* strain when either wild-type Adr1 or Adr1^c was the activator (Table 3). Efficient derepression by Adr1^c was also abolished in the double *cat8Δreg1Δ* mutant.

Adr1^c partially suppresses Cat8 dependence at genes activated by both Adr1- and Cat8: In addition to showing that Cat8 was required for the constitutivity conferred by *reg1Δ*, the results with the *cat8Δ* strains indicated that *ADH2* expression mediated by Adr1^c in the presence of Reg1 was largely independent of Cat8 (Table 3: compare Adr1^c in the Cat8 wild type and Cat8Δ rows). This was in contrast to the strong Cat8 dependence when wild-type Adr1 was the activator. Thus, Adr1^c acts independently of, and even rescues, the nonactivating *ADH2* defect of a *cat8Δ* mutation. In a previous study, we found that, at some promoters regulated by both Adr1 and Cat8 such as *ADH2*, overexpression of Adr1 partially compensates for the loss of Cat8 (BIDDICK *et al.* 2008), suggesting somewhat redundant functions for Adr1 and Cat8 at coregulated promoters. We tested whether Adr1^c in single copy could suppress the requirement for Cat8 as overexpressed wild-type Adr1

does. Expression of Adr1- and Cat8-dependent genes was measured by RT–qPCR of RNA from isogenic *adr1Δ CAT8* and *adr1Δ cat8Δ* strains with either wild-type Adr1 or Adr1^c on plasmids and grown in repressing and derepressing conditions.

The mRNA levels of several Adr1- and Cat8-dependent genes in derepressing conditions are shown in Figure 4. Table S1 has data from both repressed and derepressed conditions for Adr1- and Cat8-dependent genes, which is expressed in a manner that allows comparison of mRNA levels between genes. The dependence on Cat8 was markedly reduced for *ADH2*, *ACSI*, *ADY2*, and *JEN1* when Adr1^c was the activator compared to the strong Cat8 dependence of expression of these genes when wild-type Adr1 was the activator. These results were confirmed for *ADH2* by reporter assays (Figure S3A) and by in-gel staining of ADH activity in cell extracts (Figure S3B). Genes dependent mainly on Cat8, such as *FBP1* and *ICL1*, were unaffected by Adr1^c (Figure 4). In summary, Adr1^c efficiently suppressed the Cat8 requirement for numerous genes whose derepression is normally codependent on Adr1 and Cat8. These results are consistent with those in the previous section that documented the partial Snf1 independence of Adr1^c.

Protein synthesis is required to regulate Adr1-pSer230 levels: The importance of pSer230 and the Ser230 domain was demonstrated by the activity of Adr1^c, even in low glucose and even without its coregulator Cat8. To address the mechanism by which Ser230 phosphorylation levels are regulated, we measured phosphorylation levels of Adr1-Ser230 in the absence of protein synthesis. The amount of total and Ser230-phosphorylated Adr1 was determined by Western blotting. In the presence or absence of glucose after the

TABLE 2

Expression from an *ADRI*-dependent reporter lacking *Cat8*-binding sites

Genotype ^b	Activator ^c	β-Galactosidase activity ^a	
		R ^d	DR (8 hr)
<i>REG1</i>	Adr1	1.2 (0.7)	260 (98)
<i>REG1</i>	Adr1-S230A	21 (10)	450 (180)
<i>reg1Δ</i>	Adr1	30 (3.8)	NM ^e
<i>reg1Δ</i>	Adr1-S230A	36 (7.3)	NM

R, repressing growth conditions. DR, derepressing growth conditions.

^a UAS1 reporter activity (pHDY10). Data show average values in Miller units for at least three transformants with standard deviations in parentheses.

^b The strains are *REG1*: CKY13; *reg1Δ*: CKY5; both are *adr1Δ::kanmx*.

^c Wild-type Adr1 is carried on pKD16; Adr1^c is on pKD14 (S230A).

^d Growth conditions are described in MATERIALS AND METHODS.

^e NM, not measured.

addition of cycloheximide (Cyh), the Adr1-pSer230 and total Adr1 decreased at the same rate, suggesting that Ser230-phosphorylated Adr1 and total Adr1 turn over at the same rate in both repressing and derepressing growth conditions in the absence of protein synthesis (data not shown).

In a more complex experiment diagrammed in Figure 5, the inhibitor was added to either repressed (lanes a–c, +Cyh) or derepressed cultures (samples in lanes d and e, +Cyh), and samples were removed at various times to assay for total Adr1 and Adr1-pSer230. Control cultures lacking the inhibitor were sampled at the same time. In the absence of Cyh, the level of phosphorylation decreased 3- and 4.5-fold after 1 and 1.5 hr of derepression, respectively (compare samples in lanes a, b, and d, no Cyh). Adding glucose back into the control derepressed cultures resulted in a return to the repressed level of phosphorylation (samples in lanes c and e, –Cyh). However, if protein synthesis was inhibited in repressing conditions (left panels) before the culture was shifted to

TABLE 3

Synergistic activation of *ADH2* by Adr1^c *reg1Δ* is *Cat8* dependent

<i>ADRI</i>	Genotype ^a		<i>ADH2</i> mRNA level/ <i>ACT1</i> ^b	
	<i>CAT8</i>	<i>REG1</i>	R	DR
Wild type	Wild type	Wild type	0.0025(0.005)	2.3 (0.5)
Wild type	Wild type	Δ	0.54 (0.12)	1.3 (0.3)
Wild type	Δ	Wild type	0.0016 (0.001)	0.26 (0.02)
Wild type	Δ	Δ	0.004	0.0025
Adr1 ^c	Wild type	Wild type	0.14 (0.03)	11 (2.5)
Adr1 ^c	Wild type	Δ	1.4 (0.16)	5.6 (0.2)
Adr1 ^c	Δ	Δ	0.001 (0.0005)	0.13 (0.02)
Adr1 ^c	Δ	Wild type	0.1 (0.02)	7.2 (0.38)

R, repressing growth conditions. DR, derepressing growth conditions.

^a Strains used are *adr1Δ* strains CKY13 (*CAT8 REG1*), CKY23 (*cat8Δ REG1*), and SRY60 (*cat8Δ reg1Δ*) with plasmid pKD16 (*ADRI* wild type) or pKD14 (Adr1^c).

^b mRNA levels were measured by RT–qPCR as described in MATERIALS IN METHODS. Data are the mean of two or three replicates (standard deviation).

derepressing conditions, the relative level of Adr1-pSer230 did not decrease (compare samples in lanes a and b, +Cyh). Moreover, in these conditions, the relative level of Adr1-pSer230 did not change when glucose was added back (compare samples in lanes a and c, +Cyh). Similarly, if protein synthesis was inhibited in derepressing conditions (right panels) and then the culture was shifted to repressing conditions, the relative level of Adr1-pSer230 did not increase (compare samples in lanes d and e, +Cyh). These results suggest that synthesis of a phosphatase, a kinase, or new Adr1 is needed to modulate the ratio of Adr1-pSer230 to total Adr1 when cells encounter different glucose concentrations.

Screening for the Adr1-Ser230 kinase: A peptide representing the Ser230 region of Adr1 is an efficient substrate *in vitro* for both PKA (DENIS *et al.* 1992) and CMK (HOOK *et al.* 1999). Early studies suggested that Adr1 was inactivated *in vivo* by phosphorylation of Ser230 by PKA (CHERRY *et al.* 1989), but genetic analysis

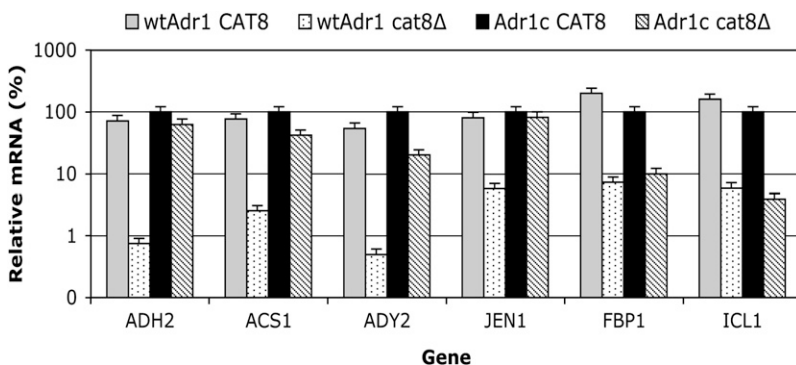


FIGURE 4.—Adr1^c suppresses *cat8Δ*-associated defects in Adr1-dependent gene expression. Cell growth is described in MATERIALS AND METHODS. RT–qPCR was performed and analyzed as described in MATERIALS AND METHODS. Strains were wild type (wt) CKY13 (*adr1Δ*) and *cat8Δ* CKY23 (*adr1Δ cat8Δ*), each carrying either wild-type *ADRI* (pKD16) or *ADRI-S230A* (Adr1^c, pKD14). Derepression was for 4 hr in 0.05% glucose–3% glycerol. The mRNA values were normalized to *ACT1* mRNA and are expressed as the percentage of the mRNA level in the wild-type *CAT8* strain with Adr1^c as the activator. The values represent the mean ± one standard deviation of three biological replicates assayed in duplicate.

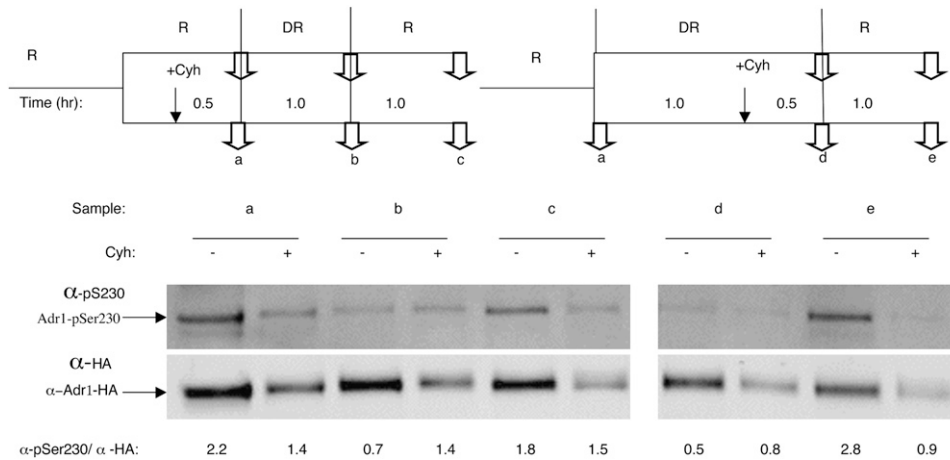


FIGURE 5.—Stability of Adr1-HA and Adr1-pSer230 in the absence of protein synthesis. Strain NKY85 was grown in complete medium containing glucose at a final concentration of 5% (R, repressing; sample lanes a–c). At the time indicated by the vertical bars (top), a portion of the culture was shifted to derepressing (DR) conditions (sample lanes d and e) by centrifugation and resuspension in low-glucose medium. In the cases indicated, glucose was added to the derepressed cultures at the times indicated by the vertical bars to reinstate repressive conditions. Cyt was added to a final concentration of 100 $\mu\text{g}/\text{ml}$ at the times indicated by a vertical arrow to a portion of the culture to inhibit protein synthesis. Samples were removed for electrophoresis and Western blotting at the points indicated by two arrows at a–e. The signal intensity was measured as described in MATERIALS AND METHODS for several biological replicates. The values shown are the mean \pm one standard deviation.

tration of 100 $\mu\text{g}/\text{ml}$ at the times indicated by a vertical arrow to a portion of the culture to inhibit protein synthesis. Samples were removed for electrophoresis and Western blotting at the points indicated by two arrows at a–e. The signal intensity was measured as described in MATERIALS AND METHODS for several biological replicates. The values shown are the mean \pm one standard deviation.

was less supportive of this model (DENIS *et al.* 1992; DOMBEK and YOUNG 1997). To determine whether Ser230 is phosphorylated *in vivo* by these protein kinases, we used yeast strains lacking all PKA activity (*tpk1 Δ tpk2 Δ tpk3 Δ yak1 Δ* and *tpk1 Δ tpk2 Δ tpk3-ts*) or all CMK activity (*cmk1 Δ cmk2 Δ cmk3 Δ*). The *tpk*-null strain was kept viable by deleting *YAK1*, encoding another protein kinase (GARRETT and BROACH 1989). This strain showed normal glucose regulation of *ADH2* (data not shown), consistent with previous studies with a *tpk1 Δ tpk2 Δ tpk3-ts* mutant (VALLARI *et al.* 1992; DOMBEK and YOUNG 1997). The triple *cmk*-null strain was viable but grew slowly, and its *ADH2* regulation was also normal (data not shown). We expected pSer230 to be absent if the mutations eliminated the Ser230 kinase, but phosphorylated Adr1 was clearly detectable in strains lacking either PKA or CMK. The relative level of Adr1-pSer230 compared to total Adr1 was measured by Western blotting in strains containing overexpressed Adr1 and was found to be altered by a factor of less than two for both PKA and CMK mutant strains (Figure 6A). The 30% reduction in the *cmk1-3 Δ* strain may be a consequence of the partial degradation of Adr1 that occurred in this strain. A similar result was obtained in a *tpk1 Δ tpk2 Δ tpk3-ts* mutant (N. KACHEROVSKY, unpublished results).

The presence of Adr1 phosphorylated on Ser230 in the *tpk*-null strain was not due to overexpression. Adr1-pSer230 was also detected in the *tpk1-3 Δ yak1 Δ* strain with single-copy Adr1 after immunoprecipitation. The immune precipitates from wild type and the quadruple mutant were analyzed by SDS-PAGE and Western blotting for Adr1. The Licor secondary antibody revealed a similar amount of Adr1 in both immune precipitates (Figure S4). The low concentration of Adr1-pSer230 in the supernatant fraction precluded its detection.

As a second method of testing for *in vivo* phosphorylation of pSer230 by PKA, we measured the relative level of Adr1-pSer230 in a strain lacking Bcy1, the regulatory subunit of PKA. The *bcy1 Δ* mutation was introduced into the same multicopy strain used to study pSer230 in the mutants described in Figures 2 and 5. The total amount of Adr1, as determined by blotting for the HA epitope on Adr1, was lower in the *bcy1 Δ* strain than in the wild-type parent, as expected, because constitutive activation of Tpk reduces expression from the *ADRI* promoter (DOMBEK 1997). We observed a slow rate of *ADH2* derepression, measured by both *in situ* staining of ADH activity and by reporter assays (Figure S5), most likely because of the lower levels of Adr1. If PKA phosphorylates Ser230 *in vivo*, we expected to see a significant increase in Adr1-pSer230 in this strain. However, the relative level of Adr1-pSer230 was unaffected in the *bcy1 Δ* mutant (Figure 6B).

We considered the possibility that Adr1-pSer230 might already be fully phosphorylated in repressing conditions (Figure S1). If so, the effect of the constitutive activation of PKA in the *bcy1 Δ* strain might be observed only in derepressing conditions, when Adr1-pSer230 decreases in the *BCY1* parent strain (Figure 2). However, loss of *BCY1* did not increase the relative level of pSer230 in either repressing or derepressing conditions (Figure 6B and data not shown). This result is unlikely to be a consequence of the reduced level of Adr1 in the *bcy1 Δ* mutant because the level of Adr1 in the multicopy *bcy1 Δ* strain is similar to the level of Adr1 in a single-copy *ADRI BCY1* wild-type strain.

In combination with the results from the PKA triple-mutant strains, the results of two different approaches suggested that PKA is not the main kinase of Adr1 Ser230 *in vivo*. Adr1-Ser230 was clearly phosphorylated in a CMK mutant, so we concluded that neither PKA nor CMK is the main Adr1-Ser230 kinase. PKA or CMK

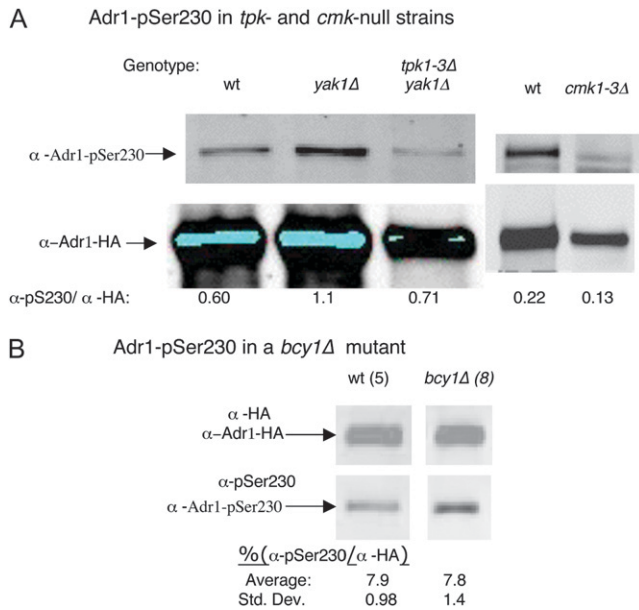


FIGURE 6.—Adr1-pSer230 phosphorylation is not significantly altered in *tpkΔ* and *cmkΔ* mutants or a *bcy1Δ* mutant. Preparation of cell extracts, electrophoresis, and Western blotting were carried as described in MATERIALS AND METHODS and in the legend to Figure 1. (A) Wild type (wt) is CMY323, *yak1Δ* is SGP400, *tpk1Δ tpk2Δ tpk3Δ yak1Δ* is SGP406, *cmk1Δ cmk2Δ cmk3Δ* is NKY108. All three strains were transformed with plasmid pNKA1, which has HA-tagged Adr1 expressed from the *ADH1* promoter on a multicopy *TRP1* plasmid. Cells were grown in *trp*⁻ synthetic medium with 5% glucose (repressing conditions). Fifty micrograms of protein extract was analyzed. (B) Wild type is NKY85 and *bcy1Δ* is NKY111, both of which are multicopy *ADRI*. Protein extracts (25 μg wild type, 100 μg *bcy1Δ*) were analyzed as described above. The numbers in parentheses indicate the number of replicates analyzed and represent both repressed (complete medium with 5% glucose) and derepressed (2, 4, and 22 hr in complete medium with 0.05% glucose–2% ethanol) cultures.

might have partial or nonspecific effects on Adr1 phosphorylation.

Because we were unable to demonstrate a major reduction in Adr1-pSer230 in mutants lacking PKA and CMK, we screened 102 viable kinase deletion mutants (as designated by the *Saccharomyces* Genome Database) for their ability to phosphorylate Adr1-Ser230. Each mutant was transformed with a high-copy plasmid overexpressing wild-type Adr1 to facilitate the screen. Immunoprecipitation with anti-Adr1 antisera, followed by Western blotting with α-pSer230, was used to detect the phosphorylated protein. No mutants that were defective in phosphorylating Adr1-Ser230 were identified (data not shown).

DISCUSSION

Although Ser230 is part of a canonical consensus sequence for PKA and is phosphorylated by PKA *in vitro* (DENIS *et al.* 1992), two different methods failed to confirm PKA as the Ser230-specific kinase. In addition, a

mutant lacking three yeast genes encoding Cmk homologs had only a minor effect on Ser230 phosphorylation. A screen of 102 viable yeast protein kinase mutants failed to identify a candidate kinase for Ser230. In the experiments to test Ser230 kinase candidates, overexpression of Adr1 was necessary to quantify the level of Adr1-pSer230. Although we cannot rule out the possibility that nonspecific phosphorylation of the overexpressed protein masked identification of the important kinase, the wild-type control with the same plasmid did not show excessive or nonspecific phosphorylation, making this explanation unlikely. Moreover, in a strain expressing normal low levels of Adr1, Ser230 phosphorylation appeared to be present at the same level in wild-type and *tpk*-null strains. *In vivo* phosphorylation of Ser230 might involve both Cmk and Tpk, but unregulated Tpk activity did not significantly increase the level of Adr1-pSer230, making this possibility less likely. Therefore, we tentatively conclude that either Ser230 can be phosphorylated by multiple protein kinases that may include PKA and CMK or its specific kinase is essential or absent from our deletion collection for another reason. Protein synthesis appeared to be necessary to change the relative level of Adr1-pSer230. This could be interpreted to indicate that an Adr1-specific protein kinase must be synthesized as cells return to repressing growth conditions or that a protein phosphatase must be synthesized *de novo* when cells are shifted from repressing to derepressing conditions.

Our results are consistent with the original hypothesis that phosphorylation of Adr1 on Ser230 plays an important role in Adr1 function by altering its ability to regulate downstream genes in a glucose-dependent manner (CHERRY *et al.* 1989). Further investigations are required to address this issue and the role of Snf1 in modulating the level of Ser230 phosphorylation. Regardless of the mechanism, regulating the level of Adr1 phosphorylated on Ser230 appears to be a major function of Snf1 in controlling the expression of Adr1-dependent genes because a nonphosphorylatable Adr1^c can activate both Adr1-dependent genes and a UAS1-containing reporter without Snf1. In contrast, wild-type Adr1 was unable to activate the same genes in the absence of Snf1. This is consistent with our interpretation that pSer230 is a major, albeit indirect, target by which Snf1 regulates Adr1 activity. The remaining Snf1 dependence when Adr1^c is the activator of genes uniquely dependent on Adr1 could be a function of other Snf1-dependent pathways that are important for activation by Adr1 (VORONKOVA *et al.* 2006) or could reflect the requirement for Snf1 in the dephosphorylation of pSer98. This site is adjacent to the DNA-binding domain of Adr1, and Ser98 phosphorylation appears to play a repressive role in Adr1 activity by reducing Adr1 promoter binding (KACHEROVSKY *et al.* 2008). Like phosphorylation of Ser230, phosphorylation of Ser98 is modulated by Snf1 activity and is reduced

in derepressing growth conditions. Whether the two modifications are causally or functionally related is under investigation.

We previously suggested that *Adr1^c* and *Snf1* act on *ADH2* expression through separate pathways (DOMBEK *et al.* 1993) on the basis of the strong synergism between *ADR1^c* and the deletion of *REG1*. The data presented here indicate that the synergism is due to *Cat8*. Thus the primary effect of *Snf1* on *ADH2* expression in a strain carrying *ADR1^c* is due to *Cat8* whose expression and activity are *Snf1* dependent. The enhanced activity of *Adr1^c* may be related to the observation that it activated genes normally codependent on *Adr1* and *Cat8* in the absence of *Cat8*. Eukaryotic promoters generally respond to different environmental signals through the action of multiple transcription factors, leading to combinatorial control of gene expression. Many *Adr1*-dependent genes are activated by other transcription factors as well. For example, the promoters of genes encoding peroxisomal proteins and the enzymes of β -oxidation bind both *Adr1* and the heterodimeric, oleate-responsive transcription factors *Oaf1* and *Pip2* (SIMON *et al.* 1991, 1992; GURVITZ *et al.* 2000, 2001; YOUNG *et al.* 2003). We are investigating whether *Adr1^c* can compensate for the loss of *Oaf1/Pip2* in the expression of peroxisomal genes, as it does for the loss of *Cat8* at *Adr1*- and *Cat8*-dependent promoters.

The finding that *Adr1^c* can suppress the requirement for *Cat8* suggests that, in spite of its low protein levels, *Adr1^c* has characteristics similar to overexpressed wild-type *Adr1*, which also escapes *Snf1* regulation (DENIS 1987) and also compensates for loss of *Cat8* at coregulated promoters (BIDDICK *et al.* 2008). *In vitro*, by electrophoretic mobility shift assays, *Adr1^c* does not have exceptional DNA-binding activity (TAYLOR and YOUNG 1990); however, it may be able to bind promoters efficiently *in vivo*, where factors such as chromatin structure and stabilization by coactivator interaction are proposed to influence *Adr1* binding (TACHIBANA *et al.* 2007; YOUNG *et al.* 2008). The wide variety of constitutive *Adr1* mutations, such as a complete deletion of amino acids 220–263 and 262–330 (COOK *et al.* 1994), suggests that not only the Ser230 phosphorylation site, but also the entire region is important for *Adr1* regulation. Our results are consistent with a model that mutations in this area affect protein structure or alter interaction with other factors (COOK *et al.* 1994). Although no *Adr1*-specific repressor has been found in spite of several genetic screens, these findings about *Adr1^c* suggest a model that can drive further investigation of the novel interaction between *Adr1* and *Snf1*.

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GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.109.103432/DC1>

Snf1 Controls the Activity of Adr1 Through Dephosphorylation of Ser230

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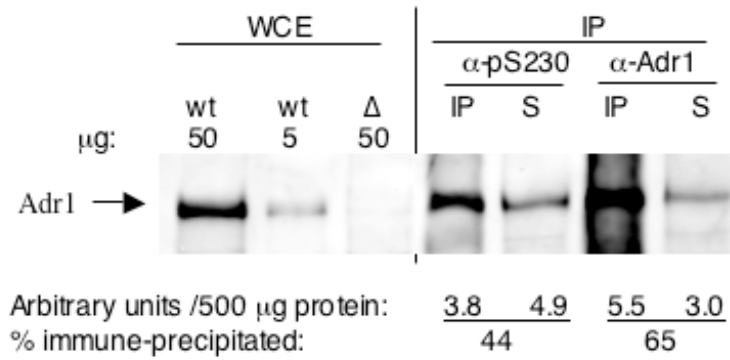
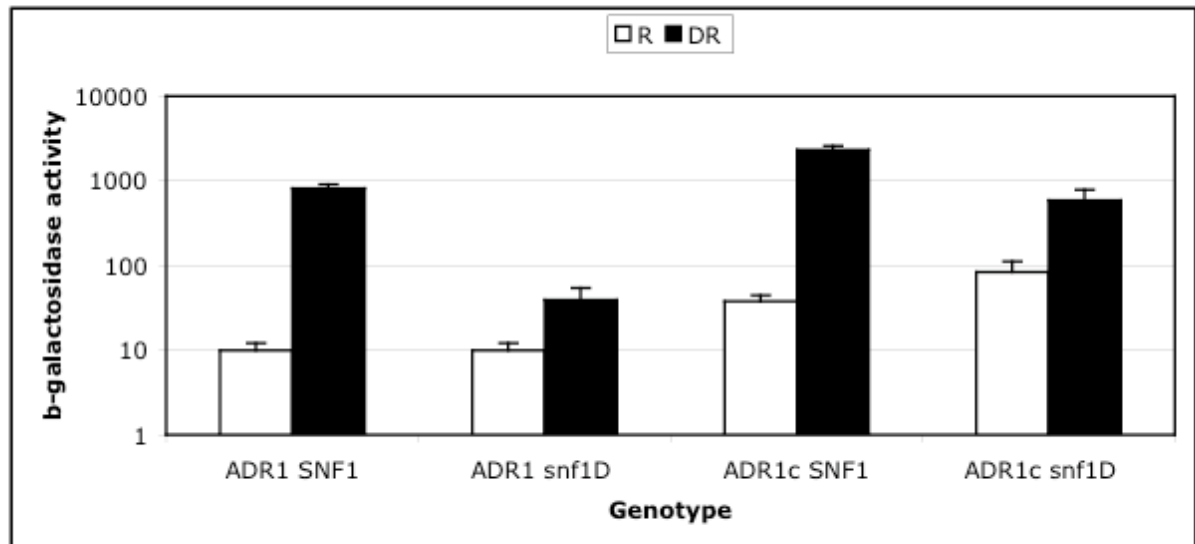


FIGURE S1.—Stoichiometry of pSer230 phosphorylation. Wildtype (wt) is NKY85 (multicopy *ADR1*); Δ is TYY204 (*adr1 Δ 1::LEU2*). Whole cell extracts (WCE) were made from cells grown in repressing conditions. 500 μ g of protein were immunoprecipitated (IP) with the antibody shown. After IP 1/5 of the pellet and 1/10 of the supernatant was analyzed by SDS-PAGE on a 6.5% gel. After Western blotting the transferred protein was identified by anti-Adr1 primary and Licor secondary antibodies.

A. *ADH2/lacZ* reporter activity

B. ADH activity assays in situ

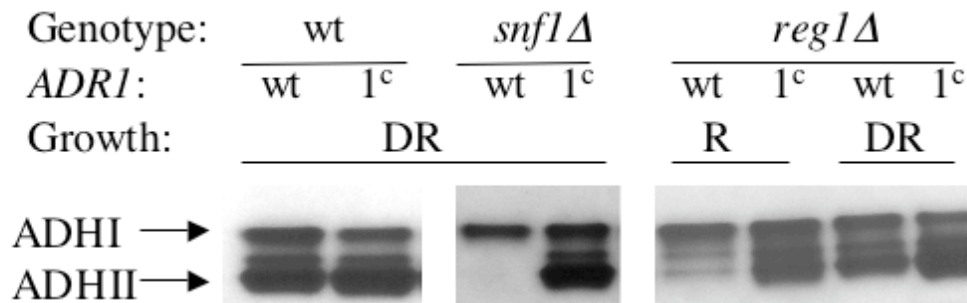
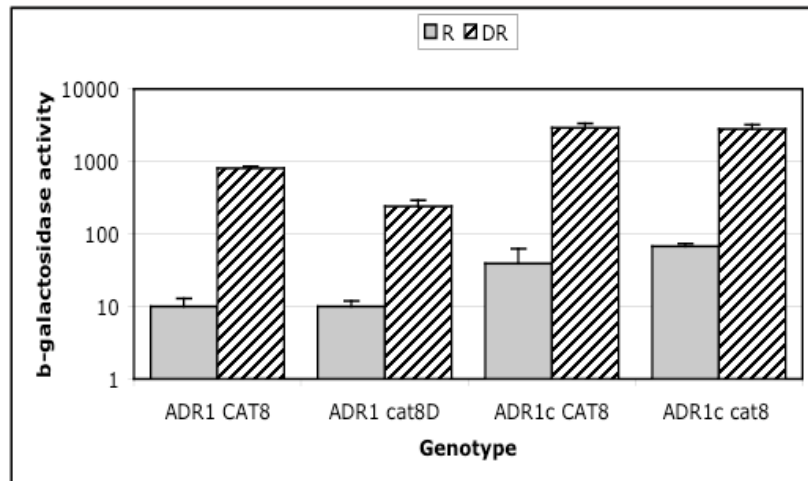


FIGURE S2.—*SNF1*-independence of *Adr1c* and synergism of *Adr1c* and *reg1Δ*. A. β -galactosidase activity. Reporter gene assays were performed using at least three transformants of strains TYY497 (*adr1ΔSNF1*) and TYY498 (*adr1Δsnf1Δ*) carrying either wildtype *ADR1* (pKD16) or *ADR1-S230A* (pKD14). β -galactosidase activity is reported in Miller units (GUARENTE 1983). The assays had an average standard deviation of about 20%. R, 5% glucose; derepressed (DR) values represent 18 hours in low glucose medium. A dilution series of the extracts was used to quantify a four-fold reduction of *ADH2* derepression in the *snf1* mutant with *Adr1c*, compared to no derepression with wildtype *Adr1* (data not shown). B. *ADH in situ* enzyme assays were performed as described in Materials and Methods. Derepression was for six hours. Wildtype (wt) is CKY13 (*adr1Δ*); *adr1Δsnf1Δ* is CKY26, and *adr1Δreg1Δ* is CKY5, each carrying either wildtype *ADR1* (pKD16) or *ADR1-S230A* (pKD14), respectively. ADHI is the constitutive fermentative isozyme of alcohol dehydrogenase encoded by *ADH1*. ADHII is the product of the *ADH2* gene. Extracts prepared from glucose-grown wild-type cells carrying either pKD16 or pKD14 did not show a band of ADHII activity (data not shown).

A. ADH2/lacZ reporter assay



B. ADH in situ enzyme activity

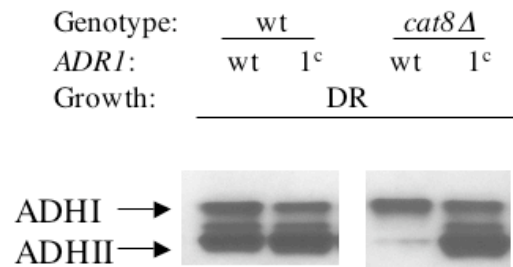


FIGURE S3.—*Adr1*^c suppresses *cat8* Δ -associated defects in *Adr1*-dependent gene expression. Cell growth is described in Materials and Methods. A. β -galactosidase activity. *CAT8* is TYY497; *cat8* Δ is TYY495 carrying either wild-type *ADR1* (pKD16) or *ADR1-S230A* (pKD14). β -galactosidase activity is reported in Miller units (GUARENTE 1983). The assays had an average standard deviation of about 20%. Derepressed (DR) values represent 20 hours in low glucose medium. B. ADH in situ enzyme assays were performed as described in Materials and Methods. Derepression was for six hours. Wildtype is CKY13 (*adr1* Δ); *cat8* Δ is CKY23 (*adr1* Δ *cat8* Δ) each carrying either wildtype *ADR1* (pKD16) or *ADR1-S230A* (pKD14).

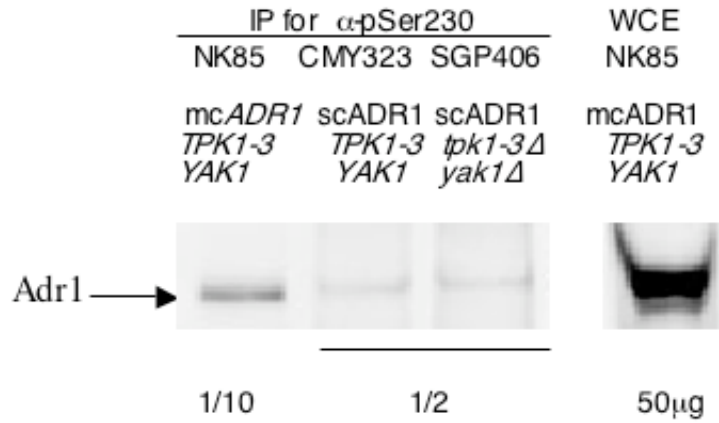


FIGURE S4. —Adr1-Ser230 in wildtype and *tpk1-3* Δ *yak1* Δ strains with single copy *ADR1*. α -Adr1 western after immunoprecipitation (IP) of whole cell extracts using anti-pSer230 antisera. IP of 500 μ g of protein from *ADR1* multicopy strain (mc*ADR1*) NK85 and 1 mg of protein from the *ADR1* single copy (sc*ADR1*) strains CKY323 (wt) and SGP406 (*yak1* Δ *tpk1-3* Δ). The fractions represent the relative amount of the IP pellet that was analyzed by SDS-PAGE on a 6.5% gel. WCE: 50 μ g of whole cell extract from strain NK85.

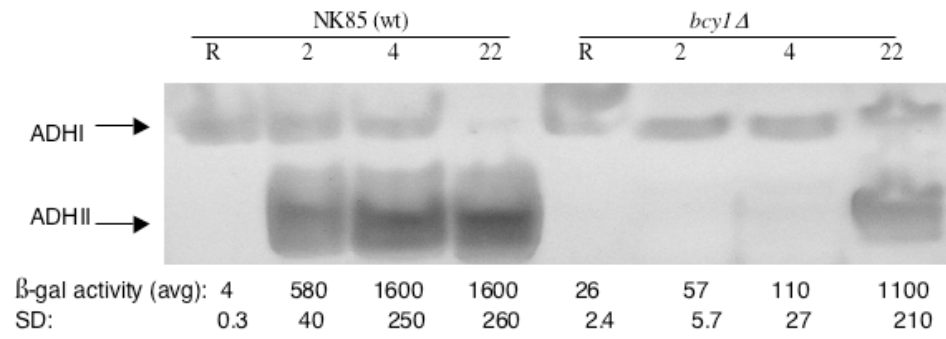


FIGURE S5. —*ADH2* expression in *BCY1* and *bcy1Δ* strains with multicopy *ADH1*. In gel assays of ADH activity and *ADH2*-β-galactosidase activity in multicopy strains NKY85 and NKY111. Cells were grown in YP-8% glucose and derepressed for the times indicated in YP-0.05% glucose-2% ethanol. Cell extracts were prepared and analyzed as described in Materials and Methods. Twenty μg of protein were analyzed.

TABLE S1

Gene expression levels with Adr1 wildtype and Adr1c activators in *CAT8* wildtype and *cat8Δ* in repressing and derepressing growth conditions

Gene	Genotype ¹	Activator ²	mRNA level/ <i>ACT1</i> ³		DR/R	Derepressed % (<i>cat8Δ</i> / <i>CAT8</i>)	
			Repressed	Derepressed		wt Adr1	Adr1c
			R	DR			
<i>AGS1</i>	<i>CAT8</i>	wtAdr1	0.013	3.3	254	3.3	42
	<i>cat8Δ</i>	wtAdr1	0.012	0.11	9		
	<i>CAT8</i>	Adr1 ^c	0.026	4.3	165		
	<i>cat8Δ</i>	Adr1 ^c	0.021	1.8	86		
<i>ADH2</i>	<i>CAT8</i>	wtAdr1	0.027	7.9	293	1.0	63
	<i>cat8Δ</i>	wtAdr1	0.026	0.082	3		
	<i>CAT8</i>	Adr1 ^c	0.14	11	79		
	<i>cat8Δ</i>	Adr1 ^c	0.1	6.9	69		
<i>ADR1</i>	<i>CAT8</i>	wtAdr1	0.016	0.059	4	42	88
	<i>cat8Δ</i>	wtAdr1	0.011	0.025	2		
	<i>CAT8</i>	Adr1 ^c	0.0079	0.041	5		
	<i>cat8Δ</i>	Adr1 ^c	0.0096	0.036	4		
<i>ADY2</i>	<i>CAT8</i>	wtAdr1	0.0063	1.3	206	1.0	20
	<i>cat8Δ</i>	wtAdr1	0.0016	0.012	8		
	<i>CAT8</i>	Adr1 ^c	0.0085	2.4	282		
	<i>cat8Δ</i>	Adr1 ^c	0.0041	0.48	117		
<i>ALD4</i>	<i>CAT8</i>	wtAdr1	0.048	1	21	47	190
	<i>cat8Δ</i>	wtAdr1	0.055	0.47	9		
	<i>CAT8</i>	Adr1 ^c	0.11	1.3	12		
	<i>cat8Δ</i>	Adr1 ^c	0.15	2.5	17		
<i>ALP1</i>	<i>CAT8</i>	wtAdr1	0.007	0.14	20	nm	nm
	<i>cat8Δ</i>	wtAdr1					
	<i>CAT8</i>	Adr1 ^c	0.0085	0.15	18		
	<i>cat8Δ</i>	Adr1 ^c					
<i>ATO3</i>	<i>CAT8</i>	wtAdr1	0.056	0.75	13	6.8	89

	<i>cat8Δ</i>	wtAdr1	0.066	0.051	1		
	<i>CAT8</i>	Adr1 ^c	0.059	0.75	13		
	<i>cat8Δ</i>	Adr1 ^c	0.066	0.67	10		
<i>CIT3</i>	<i>CAT8</i>	wtAdr1	0.0087	0.14	16	27	78
	<i>cat8Δ</i>	wtAdr1	0.00934	0.038	4		
	<i>CAT8</i>	Adr1 ^c	0.01	0.18	18		
	<i>cat8Δ</i>	Adr1 ^c	0.0073	0.14	19		
<i>CYB2</i>	<i>CAT8</i>	wtAdr1	0.035	0.28	8	75	180
	<i>cat8Δ</i>	wtAdr1	0.034	0.21	6		
	<i>CAT8</i>	Adr1 ^c	0.044	0.25	6		
	<i>cat8Δ</i>	Adr1 ^c	0.032	0.4	13		
<i>FBP1</i>	<i>CAT8</i>	wtAdr1	0.0083	1.1	133	3.6	10
	<i>cat8Δ</i>	wtAdr1	0.0034	0.04	12		
	<i>CAT8</i>	Adr1 ^c	0.0058	0.55	95		
	<i>cat8Δ</i>	Adr1 ^c	0.008	0.055	7		
<i>FDH</i>	<i>CAT8</i>	wtAdr1	0.006	0.049	8	6.9	43
	<i>cat8Δ</i>	wtAdr1	0.006	0.0034	1		
	<i>CAT8</i>	Adr1 ^c	0.01	0.35	35		
	<i>cat8Δ</i>	Adr1 ^c	0.006	0.15	25		
<i>FOX2</i>	<i>CAT8</i>	wtAdr1	0.0085	0.063	7	33	130
	<i>cat8Δ</i>	wtAdr1	0.0077	0.021	3		
	<i>CAT8</i>	Adr1 ^c	0.0083	0.08	10		
	<i>cat8Δ</i>	Adr1 ^c	0.004	0.1	25		
<i>GUT1</i>	<i>CAT8</i>	wtAdr1	0.016	0.24	15	42	160
	<i>cat8Δ</i>	wtAdr1	0.021	0.1	5		
	<i>CAT8</i>	Adr1 ^c	0.015	0.25	17		
	<i>cat8Δ</i>	Adr1 ^c	0.015	0.39	26		
<i>ICL1</i>	<i>CAT8</i>	wtAdr1	0.037	2.5	68	3.8	3.9
	<i>cat8Δ</i>	wtAdr1	0.017	0.094	6		
	<i>CAT8</i>	Adr1 ^c	0.023	1.6	70		
	<i>cat8Δ</i>	Adr1 ^c	0.0034	0.063	19		
<i>ICL2</i>	<i>CAT8</i>	wtAdr1	0.014	0.14	10	16	47
	<i>cat8Δ</i>	wtAdr1	0.011	0.023	2		

	<i>CAT8</i>	Adr1 ^c	0.0096	0.16	17		
	<i>cat8Δ</i>	Adr1 ^c	0.0024	0.075	31		
<i>JEN1</i>	<i>CAT8</i>	wtAdr1	0.0093	0.66	71	7.1	82
	<i>cat8Δ</i>	wtAdr1	0.0054	0.047	9		
	<i>CAT8</i>	Adr1 ^c	0.0088	0.82	93		
	<i>cat8Δ</i>	Adr1 ^c	0.0047	0.67	143		
<i>MDH2</i>	<i>CAT8</i>	wtAdr1	0.02	2.1	105	4.4	10
	<i>cat8Δ</i>	wtAdr1	0.02	0.093	5		
	<i>CAT8</i>	Adr1 ^c	0.023	1.8	78		
	<i>cat8Δ</i>	Adr1 ^c	0.024	0.18	8		
<i>POT1</i>	<i>CAT8</i>	wtAdr1	0.01	0.1	10	140	380
	<i>cat8Δ</i>	wtAdr1	0.009	0.14	16		
	<i>CAT8</i>	Adr1 ^c	0.015	0.13	9		
	<i>cat8Δ</i>	Adr1 ^c	0.013	0.49	38		
<i>POX1</i>	<i>CAT8</i>	wtAdr1	0.01	0.11	11	15	78
	<i>cat8Δ</i>	wtAdr1	0.011	0.017	2		
	<i>CAT8</i>	Adr1 ^c	0.015	0.23	15		
	<i>cat8Δ</i>	Adr1 ^c	0.012	0.18	15		
<i>SPG1</i>	<i>CAT8</i>	wtAdr1	0.016	0.055	3	110	210
	<i>cat8Δ</i>	wtAdr1	0.0061	0.061	10		
	<i>CAT8</i>	Adr1 ^c	0.011	0.27	25		
	<i>cat8Δ</i>	Adr1 ^c	0.017	0.58	34		
<i>SPS19</i>	<i>CAT8</i>	wtAdr1	0.014	0.098	7	38	130
	<i>cat8Δ</i>	wtAdr1	0.0073	0.037	5		
	<i>CAT8</i>	Adr1 ^c	0.032	0.15	5		
	<i>cat8Δ</i>	Adr1 ^c	0.023	0.2	9		
<i>YIL057c</i>	<i>CAT8</i>	wtAdr1	0.000024	0.036	1500	24	72
	<i>cat8Δ</i>	wtAdr1	0.000015	0.0085	567		
	<i>CAT8</i>	Adr1 ^c	0.000043	0.022	512		
	<i>cat8Δ</i>	Adr1 ^c	0.000022	0.016	727		

¹ Wild-type (wt) is CKY13 *adr1Δkanmx*; Δ is CKY19 *adr1Δkanmx cat8Δnatmx*.

² Activator is wildtype Adr1 (wtAdr1) carried on on pKD16 or Adr1c (S230A) carried on pKD14.

³ mRNA expression levels were measured by quantitative real-time PCR as described in Materials and Methods. Primer sequences are available upon request. The means of three biological replicates performed in duplicate are shown. The average standard deviation was about 20%.