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Multiple Pathways Are Co-regulated by the Protein Kinase Snf1 and the Transcription Factors Adr1 and Cat8*[S]

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***ADR1* and *CAT8* encode carbon source-responsive transcriptional regulators that cooperatively control expression of genes involved in ethanol utilization. These transcription factors are active only after the diauxic transition, when glucose is depleted and energy-generating metabolism has shifted to the aerobic oxidation of non-fermentable carbon sources. The Snf1 protein kinase complex is required for activation of their downstream target genes described previously. Using DNA microarrays, we determined the extent to which these three factors collaborate in regulating the expression of the yeast genome after glucose depletion. The expression of 108 genes is significantly decreased in the absence of *ADR1*. The importance of *ADR1* during the diauxic transition is illustrated by the observation that expression of almost one-half of the 40 most highly glucose-repressed genes is *ADR1*-dependent. *ADR1*-dependent genes fall into a variety of functional classes with carbon metabolism containing the largest number of members. Most of the genes in this class are involved in the oxidation of different non-fermentable carbon sources. These microarray data show that *ADR1* coordinates the biochemical pathways that generate acetyl-CoA and NADH from non-fermentable substrates. Only a small number of *ADR1*-dependent genes are also *CAT8*-dependent. However, nearly one-half of the *ADR1*-dependent genes are also dependent on the Snf1 protein kinase for derepression. Many more genes are *SNF1*-dependent than are either *ADR1*- or *CAT8*-dependent suggesting that *SNF1* plays a broader role in gene expression than either *ADR1* or *CAT8*. The largest class of *SNF1*-dependent genes encodes regulatory proteins that could extend SNF1 dependence to additional pathways.**

During the diauxic shift, when yeast cells deplete the glucose in the medium, the flow of metabolites changes dramatically to adapt to the use of alternative energy and carbon sources, primarily ethanol produced during fermentation. The changes in gene expression that are required for this metabolic reorganization were dramatically revealed by DNA microarray analysis (1). The expression of more than one-quarter of the yeast genome is altered when glucose is exhausted. These alterations allow the cell to utilize carbon sources other than glucose to channel metabolites into the tricarboxylic acid and glyoxylate cycles to generate energy and synthesize intermediates for gluconeogenesis. In addition, these changes allow the cell to alter its growth rate and prepare for growth arrest and stationary phase.

Numerous transcription factors and regulatory proteins are responsible for the altered transcriptional program that accompanies the depletion of glucose. Two transcription factors that play important roles during the diauxic transition are Adr1[1] and Cat8.

ADR1 was discovered as a regulatory gene that is required for expression of the glucose-repressed *ADH2*[2] gene (2, 3). *ADH2* encodes an alcohol dehydrogenase isozyme that is required for the first step in ethanol oxidation. Expression of eight other genes is dependent on *ADR1*. These include other genes important for ethanol and glycerol utilization (4–6). Six of the eight genes encode components of the peroxisomal pathway for β-oxidation of fatty acids (7–9).

CAT8 is an essential gene for growth on non-fermentable carbon sources (10). Most of the genes encoding enzymes of the glyoxylate cycle and two key enzymes of gluconeogenesis are well characterized targets of Cat8. DNA miniarray and proteomic analyses extended to 34 the number of *CAT8*-dependent genes (11). Many of the newly identified genes have roles in ethanol and lactate utilization and intracellular transport of glyoxylate and tricarboxylic acid cycle intermediates.

Many of the transcriptional changes occurring at the diauxic transition require an active Snf1 protein kinase complex (12). Snf1 is the yeast homolog of the AMP-activated protein kinase found in higher eukaryotes. Although Snf1 is present in glucose-grown cells, its activity is regulated, perhaps via phosphorylation by a Snf1 kinase kinase or by the accumulation of AMP that occurs in the cell when glucose is depleted. Its importance for aerobic growth is illustrated by the fact that *snf1* mutant cells are unable to grow on any non-fermentable carbon source, while having apparently wild-type growth properties in the presence of glucose. *SNF1* is also essential for growth on alternative sugars such as sucrose, galactose, and maltose.

Snf1-dependent phosphorylation regulates several glucose-responsive transcription factors. Phosphorylation of the repressor Mig1 leads to its export from the nucleus and derepression of glucose-repressed genes encoding enzymes required for metabolism of alternative sugars (13, 14). *SNF1* regulates both

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[S] The on-line version of this article (available at http://www.jbc.org) contains Tables I–V and additional data.

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[1] Proteins are indicated by gene names with a capital letter followed by lowercase letters (Adr1), and genotypes and gene names are capitalized in italics (*ADR1*).

[2] The abbreviations used are: ADH, alcohol dehydrogenase; ChIP, chromatin immunoprecipitation; ORF, open reading frame; UAS, upstream activation sequence.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
W303-1A/akaTYY201	*MAT***a** *ade2 can1-100 his3-11, 15 leu2-13, 112 trp1-1 ura3-1*	
W303-1B	*MATα ade2 can1-100 his3-11, 15 leu2-13, 112 trp1-1 ura3-1*	
TYY202	W303-1A with *adr1Δ1*::*LEU2*	20
TYY203	W303-1A *ADH2*::*YIpADH2/lacZ*::*TRP1*	This study
TYY204	W303-1A with *adr1Δ1*::*LEU2 ADH2*::*YIpADH2/lacZ*::*TRP1*	This study
TYY458	W303-1A with *cat8D*::*Kan*^{R} *ADH2*::*YIpADH2/lacZ*::*TRP1*	This study
TYY459	TYY458 with *adr1Δ1*::*LEU2*	This study
TYY320	TYY202 with YCpADR1-s (*TRP1*) (wild-type *ADR1*)	20
TYY324	TYY202 with pRS314 (*TRP1*) (no *ADR1*)	20
TYY390	W303-1A with *adr1Δ1*::*LEU2 snf1Δ*::*URA3*	20
TYY391	TYY390 with pRS314 (*TRP1*) (no *ADR1*)	20
TYY393	TYY390 with YCpADR1-s (*TRP1*)(wild-type *ADR1*)	20
TYY461	TYY201 with pWL21 (*IME1*/lacZ, *URA3*)	This study
TYY462	TYY202 with pWL21 (*IME1*/lacZ, *URA3*)	This study
TGY25	*MAT***a**/*MATα adr1Δ1*::*LEU2*/*adr1Δ1*::*LEU2 ade2*/*ade2 can1-100*/*can1-100 his3-11,15*/*his3-11,15 leu2-13,112*/*leu2-13, 112 URA3*/*ura3-1 trp1-1*/*TRP1*	This study
TGY27	*MAT***a**/*MATα ADR1*/*adr1Δ1*::*LEU2 ADE2*/*ade2 can1-100*/*can1-100 HIS3*/*his3-11,15 leu2-13,112*/*leu2-13, 112 ura3-1*/*ura3-1 trp1-1*/*TRP1*	This study

the activity of the Cat8 and Sip4 transcription factors and the transcription of their genes (15–18).

The interaction between Snf1- and *ADR1*-dependent gene activation is unclear. The expression of Adr1 is not *SNF1*-dependent (19), but Adr1 is unable to bind the *ADH2* promoter in the absence of Snf1 (20). Snf1 could phosphorylate and activate Adr1 for binding, or it could act on *ADR1*-dependent promoters in some other manner to allow Adr1 to bind. For example, Snf1 phosphorylates Ser-10 on histone H3, leading to acetylation of Lys-14 and activation of the *INO1* promoter (21). If Snf1 and Adr1 act in a concerted but independent manner on target genes, some *ADR1*-dependent genes might be activated independently of Snf1. Such genes could be identified by examining the overlap between *ADR1*- and *SNF1*-dependent genes using DNA microarrays.

Microarrays could help identify other transcription factors that play a role in the activation of Adr1 target genes. For example, two genes involved in ethanol metabolism, *ADH2* and *ACS1,* are co-regulated by Adr1 and Cat8 in a synergistic manner (22). The genes of β-oxidation that are *ADR1*-dependent are also regulated by oleate induction through the transcription factor Oaf1·Pip2 (8, 9). *GUT1* is regulated by Ino2 and Ino4 as well as by Adr1 (5). Thus, Adr1 acts in concert with at least three other transcription factors.

To uncover the full repertoire of *ADR1*-dependent genes and to identify those that are also *CAT8*- and *SNF1*-dependent, we performed whole genome transcriptome profiling of derepressed cells using DNA microarrays. Strains carrying deletions of *ADR1*, *CAT8*, *SNF1*, and the double mutants *adr1 cat8* and *adr1 snf1* were compared with a wild-type strain using a two-color hybridization assay. One hundred eight genes are *ADR1*-dependent in the microarray assay. Additional genes in pathways already known to be *ADR1*-dependent as well as genes encoding enzymes and proteins performing cellular functions not previously suspected of being *ADR1*-dependent were identified. Many of these genes have potential Adr1-binding sites in their promoters, and chromatin immunoprecipitation showed nine of them to be direct targets of Adr1. The *ADR1*-dependent genes encode proteins having diverse cellular functions, ranging from formate metabolism to meiosis. In a related microarray experiment in which repressed and derepressed mRNAs were compared, most of the *ADR1*-dependent genes were highly glucose-repressed. Many, but not all, *ADR1*-dependent genes are dependent for expression on *SNF1*. Despite their diversity, the *ADR1*-dependent genes have several unifying features. They are all non-essential in rich glucose medium, and many are important for growth or survival when yeasts are grown on a non-fermentable carbon source.

EXPERIMENTAL PROCEDURES

Strains—Yeast strains are listed in Table I. To create isogenic strains that differ only at the *ADR1* locus, we replaced the wild-type *ADR1* gene with *LEU2* (23) and introduced a low copy plasmid with or without *ADR1*. This was done because we observed that an *adr1*::*LEU2* strain grows better on glucose minimal media plates than its *ADR1 leu2* isogenic parent, suggesting that leucine biosynthesis may be limiting under these conditions. By using this strategy both the *ADR1* wild-type and the *adr1* mutant strains are Leu$^+$ and will not differ in gene expression because of unknown indirect effects of leucine auxotrophy. The plasmid copy of *ADR1* activates *ADH2* expression to a level similar to the level activated by the endogenous *ADR1* gene (24). Strain TYY390 was created from TYY202 by disrupting *SNF1* with a *snf1*::*URA3* fragment derived from pST70 (25). Strains TYY458 and TYY459 were created by disrupting *CAT8* in strains TYY203 (*ADR1*) and TYY204 (*adr1Δ1*::*LEU2*) with a PCR fragment generated from a KAN^R disruption cassette present in plasmid pUG6 (26).

Growth of Yeast Cultures—Yeast cultures were grown in synthetic (SM) or rich (YP) medium (27). To maintain selection for plasmids containing *TRP1,* 0.1% casamino acids was added rather than trp$^-$ drop-out solution. For repressed growth conditions, glucose was present at 2.5%. For growth in derepressing conditions, the cells were pelleted by centrifugation when they reached an A_{600} of 1.0 and resuspended in medium containing 0.05% glucose. The cells were harvested 6 h later by centrifugation and washed with 1/10 volume of cold H_2O.

DNA Microarray Analyses—Cy3- and Cy5-labeled probes were prepared from poly(A) RNA and hybridized to yeast open reading frame (ORF) microarrays as described previously (28) with minor modifications. Total yeast RNA was isolated by hot phenol/SDS-glass bead breakage, followed by ethanol precipitation (24), and treated with DNase I that had been incubated with iodoacetate to inactivate RNase activity. Poly(A)-enriched RNA was isolated using an Oligotex mRNA Purification kit (Qiagen Corp., Valencia, CA). Forward (Cy3-wild-type/Cy5-mutant) and reverse labeling (Cy5-wild-type/Cy3-mutant) comparisons were performed for each experiment using 15 pmol of Cy3 and 25 pmol of Cy5-labeled cDNAs. Three biological replicates of the *ADR1* wild-type to Δ*adr1* mutant comparison and one each of the wild-type to Δ*snf1*, Δ*snf1*Δ*adr1,* Δ*cat8,* and Δ*cat8*Δ*adr1* comparisons were done. The first two Δ*adr1* experiments utilized low density yeast ORF microarrays prepared as described previously (28). PCR products from 4608 of the smaller ORFs were spotted in duplicate onto one slide and the remaining 1536 larger ORFs were spotted in duplicate onto a second slide. The third replicate of the Δ*adr1* experiment and all subsequent experiments utilized high density microarrays that contained 6129 yeast ORFs spotted in duplicate on each slide. These first generation high density microarrays were produced by the Center for Expression Arrays in the Department of Microbiology at the University of Washington (Seattle, WA, ra.microslu.washington.edu/aboutus/about_us.html). Hybridized microarray slides from the first wild-type to Δ*adr1* comparison experiment were scanned using a Generation II Scanner from Amersham Biosciences. Slides from all subsequent experiments

TABLE II
Primers used for PCR analysis of Adr1 ChIP DNA

Primers	Sequence
CTO-ACT1A	GTATGTTCTAGCGCTTGCACCATC
CTO-ACT1B	TACCGACGATAGATGGGAAGACAG
CTO-ADH2A	TATGATCCGTCTCTCCGGTTACAG
CTO-ADH2B	GTGAACCCCATTTCTATGCTCTCC
ADY2-F-2	TGACCCGGGCTCCAAGCTACGGTTTTTACG
ADY2-R-2	ATACTCGAGGGTGTCTCTGACAGGTTGGAG
ALD4-F	CATTCTCCCGGGCCTTTAGTACCGTCCGCAC
ALD4-R	GGAAAGCTCGAGGAAGTCAATTGTCACCGAC
CIT3-F	GCTTATGGTACCGTTGAAGCCGTTATGTTGTCG
CIT3-R	TCTCCTCTCGAGGGTCATCCATTTTCAGTGTGG
ICL2-F	AGCTAAGGTACCTGATGTAGAAAGAGGTGTCAG
ICL2-R	TTATATCTCGAGCGTATGATATCGCTAGCATGA
GIP2-F	TGAAGGGGTACCGAAGTTCGTTCTATCCAACAGG
GIP2-R	AATAAACTCGAGACAAGATATCTGTACCCGTTCC
MRF1-F	TATGTTGGTACCATTTAGTTATAAAGCAAGTGGTAGC
MRF1-R	TGACAACTCGAGTTTGTAATCTCCAATTTCTTTAGCC
POX1-F	ATTATTCCCGGGCTTTAAAACCTATAATGTGC
POX1-R	AAATAACTCGAGCAGGTTAGACCTTTATAAACACTC
YIL057-F	CGTAATCCCGGGCAATGCACCCACGACTCGCGGTG
YIL057-F	TGCAAGCTCGAGGGGCAATAGAGGATTGGGACAGC
YPL276-F-1	TTACCCGGGTTGTCACCATTTGAGAATAAGC
YPL276-R-1	TCTCTCGAGCAATGATTGACAGTGCAGAGTG

were scanned using a Generation III Scanner (Amersham Biosciences) at the Center for Expression Arrays.

Raw spot intensities, local background, and spot quality estimates were extracted from microarray images using the image analysis program Dapple (29). The raw data were processed using the DNA Microarray Data Processing Pipeline Perl scripts and software developed at the Institute for Systems Biology (Seattle, WA, db.systemsbiology.net/software/ArrayProcess/index.html). Preprocessing of the data to normalize background-subtracted intensities and to calculate $\log_{10}$ ratios was performed as described previously (28). In brief, preprocessed data were subjected to several quality control checks before proceeding further with the analysis. First, the $\log_{10}$ ratio for each spot was plotted against its duplicate on the same array slide. Only data from slides having points clustered about a straight line with a positive slope of approximately 1 were further analyzed. Next, $\log_{10}$ ratios from forward and reverse labeling experiments were plotted against each other. Only data from experiments having points clustered about a straight line with a negative slope of approximately 1 were further analyzed. The data from each experiment were then merged and filtered using Dixon's outlier rejection test. Data from all three biological replicates of the wild-type to Δ*adr1* mutant comparison were merged into one data set.

To identify genes showing significantly different levels of mRNA, a maximum likelihood error model was produced for each data set using the program VERA, and this model was applied to that data set using the program SAM (www.systemsbiology.org/VERAandSAM). SAM uses the model produced by VERA to calculate a maximum likelihood ratio statistic (λ) and a $\log_{10}$ ratio (muRatio) that is corrected for additive and multiplicative errors that arose during the course of the experiment. Each expression ratio presented in this paper is the anti-log of the muRatio. A muRatio of 0.3 or greater, representing a 2-fold or greater difference in mRNA level, was arbitrarily chosen as one criterion of significance. A λ cutoff value of 44.0 for the low density microarrays and a cutoff value of 27.5 for the high density arrays were chosen as second criteria of significance. These λ cutoff values were determined from the distribution of λ values across all genes in a self-hybridization experiment for each type of microarray and represent the λ value below which 95% of the genes fall in the control experiment. Higher λ values eliminated many genes already known to be *ADR1*-dependent. For these control experiments, Cy3-labeled cDNA was prepared from the poly(A) RNA of derepressed wild-type cells and hybridized against Cy5-labeled cDNA prepared from the same poly(A) RNA.

Promoter-binding Site Analyses—Upstream sequences up to 600 bp from the translational start site of each gene were retrieved and analyzed using the Web-based RSA Tools (30) (embnet.cifn.unam.mx/rsa-tools). Transcription factor-binding sites were identified using the dna-pattern tool with the following sequence patterns: Adr1-binding site, DYGGRG, and exact matches to the UAS1-like sequence, CYCCRHN{2,36}DYGGRG (31); Cat8-binding site, CSRE, CCDNHN{3}CCG (11); Oaf1-Pip2-binding site, ORE, CGGN{15,18}CCG (32). The DNA-pattern tool was unable to search for UAS1-like sequences with random mismatches. Therefore, we developed the Perl script seekw and used it to search for UAS1-like sequences allowing for one mismatch anywhere in the consensus. Both strands of all upstream sequences were searched for matches to each of these patterns.

Construction of Reporter Plasmids—The base plasmids were pLG669Z (33) and pLG669Z(K) (34). Promoter fragments from the desired ORFs were amplified from genomic DNA using primers flanking the region of interest. The PCR products were either cloned as *Xho*I-*Xma*I or *Xho*I-*Kpn*I restriction fragments directly into pLG669Z or pLG669Z(K), respectively.

Chromatin Immunoprecipitation Analysis—Chromatin immunoprecipitation was performed as described (20), except that cross-linking was with dimethyl adipimidate for 45 min (35) followed by formaldehyde treatment for 15 min. Primers used for PCR are listed in Table II.

RESULTS

One Hundred and Eight Genes Are ADR1-dependent for Expression—Fig. 1 illustrates the functional categories of 108 genes whose expression is reduced 2-fold or more in the absence of *ADR1* when cells are grown in depressing medium. The major categories of functionally annotated genes are non-fermentative carbon metabolism, peroxisome biogenesis and β-oxidation, amino acid transport and metabolism, sporulation and meiosis, and transcriptional regulation and signal transduction. Thirteen genes are annotated but do not fall into any one of these categories. Thirty percent of the *ADR1*-dependent genes have unknown functions, similar to the fraction of genes of unknown function in the whole yeast genome. None of the *ADR1*-dependent genes are essential for growth in rich medium containing glucose as a carbon source, and none of the annotated genes are involved in cell cycle control. This was expected because *adr1* mutants have no obvious growth defect in rich medium.

ADR1-dependent Genes Channel Metabolites into Acetyl-CoA and NADH Production—Fig. 2 illustrates the major pathways of carbon utilization in yeast. The genes that are expressed in an *ADR1*-dependent manner in derepressed conditions are shown in *red boxes* with their positive expression ratio, wild-type/*adr1*. Negative values in *green boxes* indicate genes whose expression is higher in the absence of *ADR1* than in its presence.

ADR1-dependent genes figure prominently in pathways leading from ethanol, glycerol, lactate, and the oxidation of fatty acids to the formation of acetyl-CoA, generating NADH in the process. Formate metabolism is also apparently *ADR1*-dependent based on the strong dependence of *FDH1* and *FDH2* on *ADR1* for expression. Formate and propionate, although un-

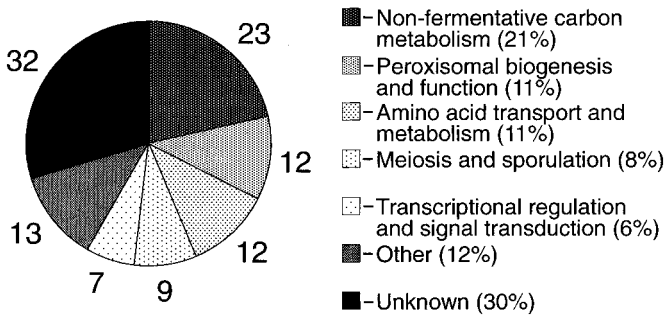

FIG. 1. **Functional categories of *ADR1*-dependent genes.** *ADR1*-dependent genes that were functionally annotated in the SGD data base were categorized based on their geneontology annotations (genome-www.stanford.edu/Saccharomyces/help/gotutorial.html). Genes falling into categories with three or fewer members were grouped into the category *Other*. Genes of unknown function were categorized based on homologies to genes of known function. Remaining genes were grouped together in the category *Unknown*. Each section of the pie chart represents a functional category, and the *number* next to it is the number of members of that category. The percentage of the total 108 *ADR1*-dependent genes present in each category follows the description of each category in the *key* next to the chart.

able to support yeast growth, can be co-metabolized with growth-limiting amounts of glucose to produce NADH, thus providing reducing equivalents that increase the growth potential of the cell (36, 37). Table III lists the *ADR1*-dependent genes of known function, their degree of *ADR1* dependence, and their biochemical or physiological function based on information available on the SGD or the MIPS web sites. Regulation of the genes in Table III is described in more detail under "Discussion."

ADR1 and β-Oxidation—The pathway of β-oxidation in yeast is shown in Fig. 3. The entire pathway of β-oxidation in yeast takes place in peroxisomes, membrane-bounded organelles whose size and number increase in response to the presence of fatty acids in the medium (38). The microarray data extend to at least 12, the number of genes encoding peroxisomal proteins that are dependent on *ADR1* for expression in the absence of oleate (Table III). Many of the *ADR1*-dependent genes scored high in a genomic profile of genes involved in peroxisome assembly or function (39) and were also identified in a search for oleate-inducible, *OAF1-PIP2*-dependent genes (40, 41). In addition to β-oxidation, uptake of fatty acyl-CoA by peroxisomes appears to be *ADR1*-dependent. *PXA1* and *PXA2* encode subunits of the ABC transporter responsible for uptake of long chain fatty acyl-CoA derivatives into peroxisomes (42). In the array data, *PXA1* showed a 5.7-fold dependence on *ADR1*. Expression of *PXA2* may also require *ADR1*, showing a 3-fold dependence. However, the significance of this dependence requires further testing because the λ value for these data was 43, just below the cutoff of 44. Medium chain fatty acids are converted into their acyl-CoA derivative by a peroxisomal acyl-CoA ligase encoded by *FAA2*. Like *PXA2*, *FAA2* may also require *ADR1* for expression, showing a 9.8-fold dependence. However, the λ statistic associated with this value was also below the established cutoff. An ORF of unknown function, *YMR018W*, encodes a protein that is homologous to *PEX5*, a pts1 receptor. Pts1 (peroxisomal targeting signal) receptors bind polypeptides that are translocated into peroxisomes (38). *YMR018W* is expressed in an *ADR1*-dependent fashion. Its role in peroxisome biogenesis has not been investigated. Another gene displaying *ADR1* dependence in the array data is *PEX11* which encodes a peroxin, a class of proteins that play a role in peroxisome proliferation (9).

Sporulation, Meiosis, and Replication/Recombination—An unexpected category of *ADR1*-dependent genes encodes proteins involved in meiosis and sporulation (Table III). This group includes two genes encoding transcription factors expressed early in meiosis, *IME1* and *SLZ1*. Other *ADR1*-dependent genes affecting meiosis or sporulation are *DMC1*, a gene important for meiotic recombination, *SPO20*, *SPR6*, *BNS1*, *CSM4*, and *ADY2*. Finding sporulation-specific genes that are expressed in an *ADR1*-dependent fashion in haploid cells is surprising because most have no known function other than in the sporulation of diploid cells, and until now, *ADR1* has not been implicated in meiosis or sporulation. Perhaps these genes have unknown functions in haploid cells during glucose starvation.

Amino Acid Metabolism—Another cohort of *ADR1*-dependent genes is involved in the transport or metabolism of amino acids (Table III). Five genes encode amino acid or peptide transporters. Two ORFs, *YLR126C* and *YDR111C*, encode putative transaminases that are identified only by homology to known glutamine amido-transferases and aspartate amino transferases, respectively, enzymes that are important for shuttling oxaloacetate between the mitochondrion, cytosol, and peroxisome. *BAT1* and *ARO9* encode branched-chain and aromatic amino acid transaminases, respectively. The expression of genes encoding enzymes that catabolize amino acids allows the cell to respond to starvation conditions by utilizing amino acids for gluconeogenesis and energy production.

Regulatory Genes—In addition to *IME1* and *SLZ1*, two other genes, *TEC1* and *NRG1,* encoding putative transcription factors, are expressed in an *ADR1*-dependent manner. Their molecular functions are unknown, but they have been implicated in pseudohyphal growth and Snf1-dependent glucose repression, respectively (43, 44). Two putative protein phosphatase regulatory subunits, *GIP2* and *RRD1,* also appear to be expressed in an *ADR1*-dependent manner (Table III). *GIP2* appears to play a role in regulating glycogen accumulation, and *RRD1* appears to be involved in the response to osmotic stress (45, 46).

Genes Whose Expression Is Enhanced in the Absence of ADR1—A significant number of genes appear to be up-regulated in the absence of *ADR1* in derepressed growth conditions (Table I, Supplemental Material). Interestingly, some of these genes encode enzymes that function in biosynthetic pathways that might be thought of as opposing the pathways dependent on *ADR1* for expression. For example, two genes encoding enzymes required for fatty acid synthesis, *OLE1* and *FAS1*, are up-regulated in the absence of *ADR1*, as is the gene, *ACH1*, encoding acetyl-CoA hydrolase. Several genes encoding glyoxylate cycle enzymes, *CIT2, ICL1*, and *MLS1*, are significantly up-regulated in the absence of *ADR1* (Fig. 2), as is *INO1*, encoding the enzyme catalyzing the first step in phosphoinositide biosynthesis. Increased expression of these genes could reflect tight coordinate regulation of the opposing catabolic-biosynthetic pathways, or could be an indirect consequence of the loss of Adr1.

ADR1-dependent Genes Are Glucose-repressed—ADR1-dependent genes should be expressed only after glucose has been exhausted from the medium because Adr1 is unable to bind chromatin in repressed growth conditions (20, 47). To determine whether glucose repression is a common feature of the genes identified as *ADR1*-dependent for expression, we co-hybridized labeled cDNAs derived from cell populations growing in repressed and derepressed growth conditions.

In general there is good agreement between our data and the data derived from the diauxic shift experiment (1). The genes that show marked derepression in both sets of data include those encoding many of the enzymes of gluconeogenesis, the

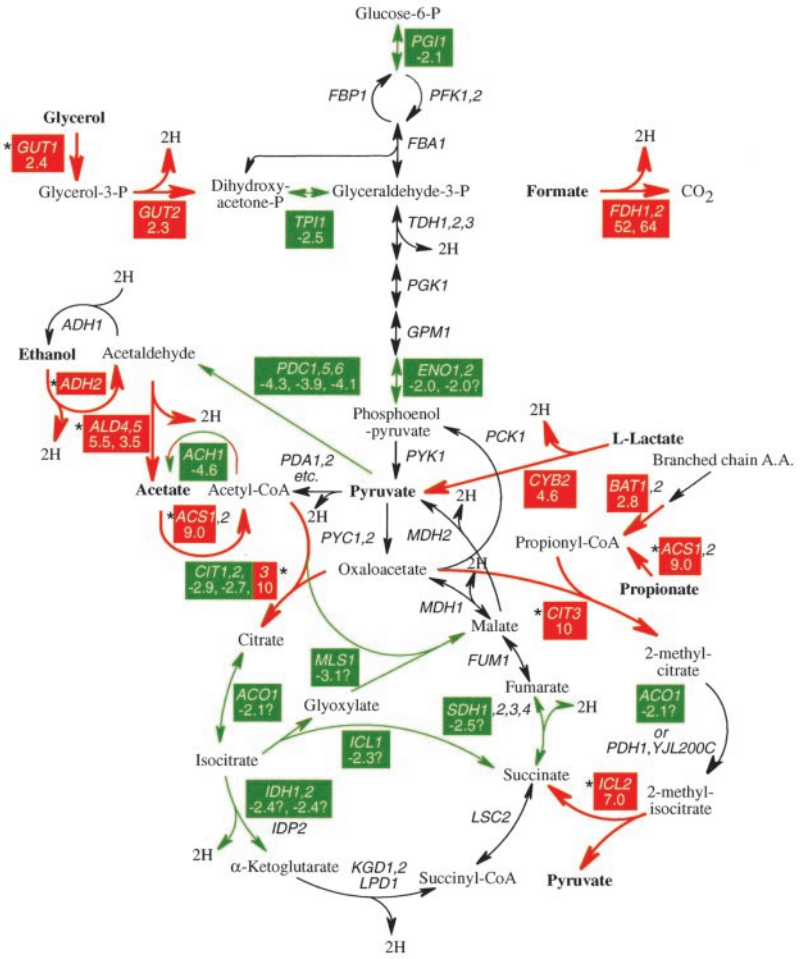

FIG. 2. **Influence of *ADR1* on expression of genes involved in non-fermentable carbon metabolism.** Pathways of non-fermentable carbon metabolism were constructed based on information taken from the literature (1, 37, 59, 60, 65). Located *below* each gene label is the fold change in gene expression. Positive values and *red color* indicate down-regulated expression in *adr1* mutant cells. Negative values and *green color* indicate up-regulated expression. A *question mark* next to a value indicates that its associated λ statistic is below the cutoff value. Most genes in *black type* showed no significant change in expression. No data were obtained for *PDA2* and *PYK1*. *ADH1* is uncolored because its expression ratios were considered unreliable due to cross-hybridization with *ADH2* message. Genes marked by an *asterisk* have promoters that have been shown previously by ChIP analysis or in the current study to bind Adr1 *in vivo*.

glyoxylate cycle, and ethanol, glycerol, lactate, formate, and fatty acid metabolism. The complete data set is given in Table I of the Supplemental Material.

Table IV shows the 40 genes that have the highest expression ratio when comparing derepressed *versus* repressed growth conditions. Among this group, 19 are at least 2-fold dependent on *ADR1* for expression. Among a larger group of 100 genes that are highly derepressed, 38 are *ADR1*-dependent for expression. The genes that encode enzymes required for the metabolism of non-fermentable carbon compounds, especially ethanol and fatty acids, are the most prominent in this group. In the group of 108 *ADR1*-dependent genes, over 80% are at least 2-fold glucose-repressed.

ADR1 and SNF1 Dependence—Another characteristic of previously identified *ADR1*-dependent genes is dependence on *SNF1* for derepression (2, 48, 49). To characterize the *SNF1* dependence of the genes identified as *ADR1*-dependent on the microarrays, labeled cDNAs derived from isogenic wild-type and *snf1* mutant strains grown in derepressing conditions were co-hybridized on DNA microarrays and analyzed. A related experiment was performed using labeled cDNAs from a wild-type strain and a double *adr1snf1* mutant to confirm the degree of *SNF1* dependence of the *ADR1*-dependent genes.

Many *ADR1*-dependent genes are also *SNF1*-dependent for expression (Table IV and Table I, Supplemental Material). Moreover, the expression ratios for these genes in derepressing conditions are very similar in the single *snf1* mutant and the double *snf1adr1* mutant, consistent with a requirement for *SNF1* to act through *ADR1* to activate *ADR1*-dependent gene expression. The exceptions to this generalization include several genes that are highly *ADR1*-dependent, such as *POT1*, *POX1*, and *CIT3*. Expression of *POT1* was *SNF1*-dependent during oleate-inducing conditions in a previous study (49), but it did not appear to be *SNF1*-dependent in our microarrays. In summary, many of the genes identified by DNA microarrays as *ADR1*-dependent for expression have two additional properties that are expected based on previous studies of a small number of *ADR1*-dependent genes: they are glucose-repressed and dependent on *SNF1* for derepression.

Properties of SNF1-dependent Genes—Over four hundred genes have a wild-type/*snf1* expression ratio in depressing conditions of two or greater (Table I, Supplemental Material). Twenty nine of 40 of the most highly glucose-repressed genes are *SNF1*-dependent for derepression (Table IV).

The functional classification of the *SNF1*-dependent genes is shown in Fig. 4. The largest class of annotated *SNF1*-dependent genes functions in transcription or signal transduction pathways. The next largest class of *SNF1*-dependent genes is involved in non-fermentative carbon metabolism. Transition from growth in high glucose to growth in low glucose does not seem to trigger a major stress response because few of the *SNF1*-dependent genes are in the heat-shock protein family. Two particularly interesting categories of *SNF1*-dependent genes are transporters (Table II, Supplemental Material) and regulatory genes (Table III, Supplemental Material). The *SNF1*-dependent expression of these genes is described in more detail under "Discussion."

Although many glucose-repressed genes are Adr1- and Snf1-dependent for derepression, there is a significant number of *SNF1*-dependent genes that is not glucose-repressed. The best

TABLE III
Functional classification of ADR1-dependent genes

Gene	Ratio	Function
Non-fermentative carbon metabolism		
FDH2[a]	64	Formate dehydrogenase
FDH1	52	Formate dehydrogenase
CIT3[a]	10	Citrate synthase
YML131W	9.1	Quinone oxidoreductase homolog
ACS1[b]	9.0	Acetate-CoA ligase
ADH5	8.2	Alcohol dehydrogenase
GLO4	7.7	Hydroxyacylglutathione hydrolase
ICL2[a]	7.0	2-Methylisocitrate lyase
ADH2[b]	(6.8)	Alcohol dehydrogenase
DIC1	6.3	Dicarboxylate transport
ALD4[a]	5.5	Aldehyde dehydrogenase
CYB2	4.6	L-Lactate dehydrogenase
YPL201C	4.5	Glycerol metabolism?
YPL113C	4.4	Lactate dehydrogenase homolog
ALD5	3.5	Aldehyde dehydrogenase
YCP4	3.4	Flavodoxin
OAC1	3.0	Oxaloacetate transport
YGR043C	2.6	Transaldolase homolog
YHL008C	2.6	Formate/nitrite transport
GUT1[b]	2.4	Glycerol kinase
MSS2	2.4	Cox1 pre-mRNA splicing factor
GUT2	2.3	Glycerol-3-P dehydrogenase
CTP1	2.1	Citrate transport
Peroxisome biogenesis and β-oxidation		
POX1[a]	55	Acyl-CoA oxidase
SPS19	17	2,4-Dienoyl-CoA reductase (NADPH)
CTA1[b]	16	Catalase
FOX2	16	3-Hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase
POT1[b]	14	Acetyl-CoA C-acyltransferase
YMR018W	6.5	Pex5 homolog; putative pts1 receptor
PXA1	5.7	Peroxisome ABC transporter
IDP3	5.0	Isocitrate dehydrogenase ($NADP^+$)
DCI1	4.7	Dodecenoyl-CoA δ-isomerase
PEX11	3.3	Peroxisomal membrane protein
YOR389W	2.7	Pex21 interaction by two-hybrid
PCD1	2.2	Peroxisomal nudix hydrolase
Meiosis and sporulation		
ADY2[a]	20	Transporter, nitrogen utilization
YPL033C	15	Meiosis
DMC1	7.0	Meiotic recombination
ATO3	6.4	Ammonia transport/Ady2 homolog
SPO20	5.6	Pro-spore membrane γ-SNARE
BNS1	3.2	Meiosis
SPS4	3.1	Meiosis
CSM4	3.0	Chromosome segregation meiosis
SPR6	2.1	Sporulation
Amino acid transport and metabolism		
YLR126C	7.7	Gln amidotransferase motif
LEU1	6.3	Leucine metabolism
ALP1	4.9	Amino acid transport
BAG7	3.8	General amino acid permease
CAR2	3.6	Arg metabolism
SSU1	3.3	Sulfite transport
YDR111C	3.2	Asp aminotransferase homolog
ARO9	2.8	Aromatic amino acid aminotransferase
BAT1	2.8	Branched amino acid aminotransferase
PUT4	2.8	Neutral amino acid transport
PTR2	2.5	Peptide transport
DAL3	2.2	Allantoin Met/ureido metabolism
Transcriptional regulation and signal transduction		
YDL156W	4.6	Tup1 homolog
IME1	4.5	Meiosis transcription factor
SLZ1	4.3	Meiosis transcription factor
NRG1	3.6	Glucose repression/invasive growth
GIP2[a]	3.6	Glc7-regulator
TEC1	2.2	TEA/ATTS family
RRD1	2.1	PP-2A regulator
Other		
ETR1[a]	3.2	Dodecenoyl-CoA δ isomerase, Fatty acid biosynthesis
FOB1	2.9	rDNA recombination
DIA3	2.6	Acid phosphatase, pseudohyphal growth
DBR1	2.6	RNA lariat debranching enzyme
ECM8	2.5	Cell wall organization and biogenesis
GSY1	2.4	Glycogen synthase
TIR1	2.4	Structural constituent of cell wall
TRF4	2.4	Mitotic chromosome condensation
GPT2	2.1	Glycerol-3-phosphate O-acyltransferase, phospholipid Biosynthesis
YPC1	2.1	Ceramidase
BTN2	2.1	Regulation of pH

[a] Promoters were shown to bind Adr1 in this study.
[b] These are genes whose promoters are known by ChIP analysis to bind Adr1 *in vivo* (20).

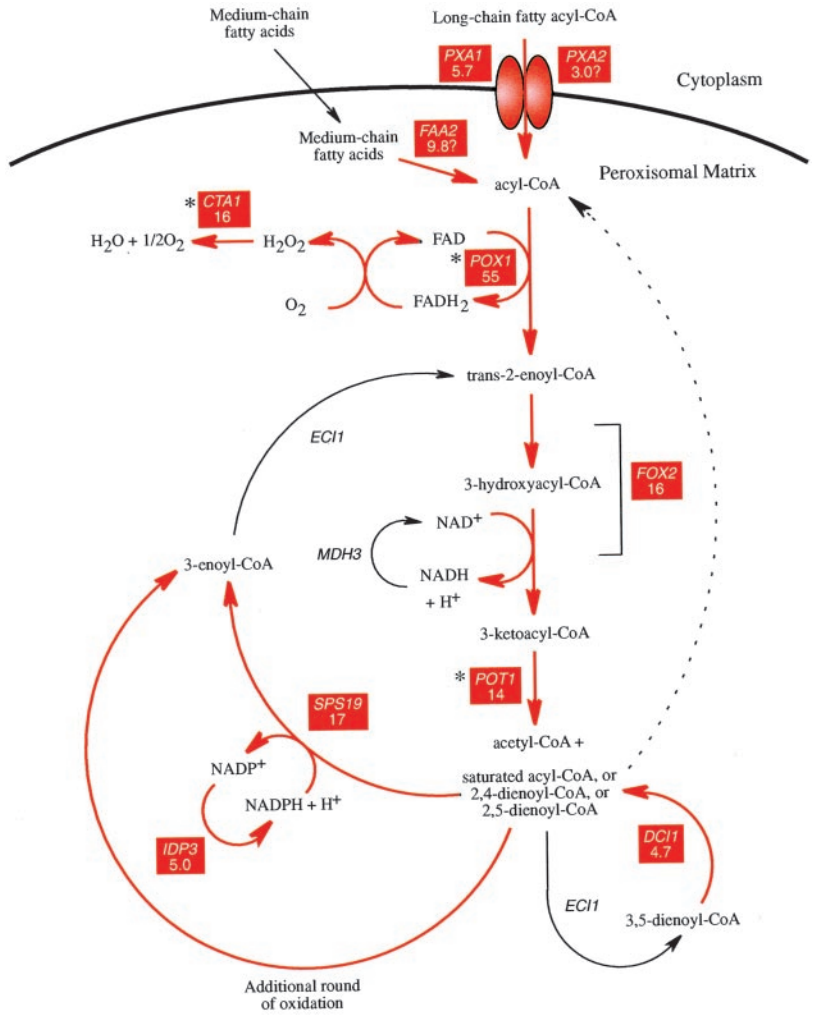

FIG. 3. **Influence of *ADR1* on expression of genes involved in β-oxidation of fatty acids.** The β-oxidation pathway was adapted from the review of fatty acid metabolism in *S. cerevisiae* by Trotter (66). Located below each gene label is the fold change in gene expression. Positive values and *red color* indicate down-regulated expression in *adr1* mutant cells. A *question mark* next to a value indicates that its associated λ statistic is below the cutoff value. All genes in *black type* showed no significant change in expression. Genes marked by an *asterisk* have promoters that have been shown previously by ChIP analysis or in the current study to bind Adr1 *in vivo*. The *dotted arrow* indicates that saturated acyl-CoA products of β-oxidation become the substrates for another round of β-oxidation.

characterized of these genes is *INO1* (Table I, Supplemental Material), encoding inositol-1-phosphate synthase, the first enzyme required for phosphoinositide biosynthesis. *INO1* induction is dependent on the Snf1 protein kinase, and repression is dependent on Reg1/Glc7 (21, 50–52). Other examples include six genes encoding histones: *HTA1*, *HTA2*, *HTB1*, *HTB2*, *HHF1*, and *HHF2* (wild-type/mutant ~3–5; Table I, Supplemental Material) and many genes involved in metal ion homeostasis (Table II, Supplemental Material). These effects could be an indirect consequence of the growth-defective phenotype of the *snf1* mutant after glucose depletion. However, a *cat8* mutant is also growth-defective after glucose depletion, but it does not affect the expression of the same subset of yeast genes (see below and Table IV, Supplemental Material). This suggests that the effect of *SNF1* is not caused by general growth defects.

Snf1 May Have a Negative Role in Gene Expression—The DNA microarray data suggest that Snf1 may play a role in down-regulating gene expression when glucose is exhausted. Many glycolytic genes are more highly represented in mRNA isolated from the *snf1* mutant than in mRNA isolated from its wild-type parent (Table I, Supplemental Material). We confirmed that one glycolytic enzyme, ADHI, is present at higher than wild-type levels in the *snf1* mutant by analyzing alcohol dehydrogenase activity using non-denaturing PAGE.[3] Thus, the elevated *ADH1* mRNA that is detected in the *snf1* mutant by the microarray is functional *in vivo*. As described above, these effects could be an indirect consequence of the growth-defective phenotype of the *snf1* mutant after glucose depletion, but they are not observed in a *cat8* mutant strain in the same conditions.

CAT8-dependent Genes—Several of the genes encoding enzymes that metabolize ethanol and lactate share a dependence on *ADR1* and *CAT8* for derepression (Table III) (11). To identify additional genes that are co-regulated by *ADR1* and *CAT8,* we co-hybridized cDNAs isolated from wild-type and *cat8* mutant cells under derepressing conditions. To identify genes cooperatively regulated by both *ADR1* and *CAT8,* a double *adr1cat8* mutant was also examined.

Over 200 genes are at least 2-fold *CAT8*- or *ADR1CAT8*-dependent in the microarray experiments. Among the 40 most highly glucose-repressed genes, 17 are at least 2-fold dependent on *CAT8* for expression (Table IV). Because the binding site or CSRE for *CAT8* is known, we could determine which of these genes are likely to be direct targets of *CAT8* regulation by examining their promoters for the CSRE motif. This analysis identified 70 genes that contained CSREs within 600 nucleotides (650 nucleotides in the case of *JEN1*) of the initiation codon. The *CAT8*-dependent genes that lack CSRE elements may be affected indirectly by the absence of *CAT8* or they could contain non-consensus CSRE motifs in their promoters. Alternatively, they may contain a CSRE element further upstream of the ATG than 600 nucleotides.

Fifty new *CAT8*-dependent genes that contain CSREs within

[3] E. T. Young, unpublished data.

TABLE IV
Expression ratios of the 40 most highly glucose repressed genes
Expression ratios in boldface type indicate a significantly higher level of expression based on a λ statistic that is above the significance threshold.

ORF	Gene	DR/R	*ADR1/adr1*	*SNF1/snf1*	*CAT8/cat8*
YLR377C	*FBP1*	**130**	0.8	**36**	**7.4**
YKL217W	*JEN1*	**98**	3.3	**28**	**2.1**
YGR236C	*SPG1*	**92**	**15**	**5.0**	0.5
YKR097W	*PCK1*	**92**	1.4	**180**	**3.6**
YJR095W	*SFC1*	**78**	1.1	**170**	**6.2**
YIL057C	*YIL057C*	**77**	**16**	**8.9**	0.8
YMR107W	*YMR107W*	**75**	2.3	**18**	0.5
YCR010C	*ADY2*	**72**	**20**	**32**	**9.3**
YDR384C	*ATO3*	**55**	**6.4**	**2.0**	**4.6**
YPL276W	*FDH2*	**50**	**64**	**5.1**	1.8
YPR001W	*CIT3*	**50**	**10**	1.4	0.7
YGL205W	*POX1*	**44**	**55**	0.8	**2.3**
YPR002W	*PDH1*	**44**	2.1	1.1	1.0
YAL054C	*ACS1*	**43**	**9.0**	**30**	**3.5**
YOR388C	*FDH1*	**35**	**52**	**4.6**	1.8
YKL187C	*YKL187C*	**34**	2.7	**6.2**	**3.2**
YDR256C	*CTA1*	**34**	**16**	**7.6**	**3.3**
YKR009C	*FOX2*	**33**	**16**	**2.4**	1.5
YGR067C	*YGR067C*	**33**	0.9	**39**	**7.1**
YNL195C	*YNL195C*	**29**	**3.0**	**7.7**	0.8
YER065C	*ICL1*	**28**	0.4	**77**	**20**
YER024W	*YER024W*	**26**	0.6	**5.6**	**2.0**
YGR243W	*YGR243W*	**26**	2.8	**6.1**	0.6
YAR035W	*YAT1*	**25**	0.9	**7.4**	1.5
YMR206W	*YMR206W*	**25**	1.2	**5.9**	1.0
YIL160C	*POT1*	**22**	**14**	1.5	0.4
YPR150W	*YPR150W*	**22**	**4.3**	**2.0**	1.3
YLR174W	*IDP2*	**21**	0.9	**16**	**2.5**
YLR126C	*YLR126C*	**21**	7.7	1.5	1.0
YBR050C	*REG2*	**21**	1.5	**42**	**5.0**
YEL008W	*YEL008W*	**21**	1.1	**7.5**	1.5
YPR006C	*ICL2*	**21**	**7.0**	**4.0**	**2.1**
YHL032C	*GUT1*	**20**	**2.4**	**6.8**	**2.3**
YHR139C	*SPS100*	**19**	0.5	1.0	0.7
YPR151C	*YPR151C*	**18**	**3.8**	1.9	1.2
YLR267W	*BOP2*	**17**	0.4	0.9	0.1
YNR002C	*FUN34*	**16**	1.0	1.3	0.6
YNL009W	*IDP3*	**15**	**5.0**	**2.0**	1.6
YNL013C	*YNL013C*	**15**	3.0	1.6	0.1
YER179W	*DMC1*	**14**	**7.0**	1.8	0.9

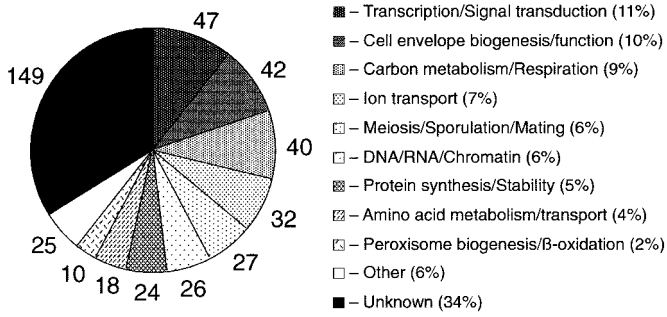

FIG. 4. **Functional categories of *SNF1*-dependent genes.** *SNF1*-dependent genes that were functionally annotated in the SGD data base were categorized based on geneontology annotations (genome-www.stanford.edu/Saccharomyces/help/gotutorial.html). Genes falling into categories with 10 or fewer members were grouped into the category *Other*. The remaining unannotated genes were grouped together in the category *Unknown*. Each section of the pie chart represents a functional category, and the number next to it is the number of members of that category. The percentage of the total 439 *SNF1*-dependent genes present in each category follows the description of each category in the *color key* next to the chart.

600 nucleotides of the ATG are listed in the 1st column of Table IV of the Supplemental Material. We confirmed the *CAT8* dependence reported earlier for 18 genes including all of the genes encoding major glyoxylate cycle enzymes (Table IV, Supplemental Material, 2nd column) (11, 15). Ten of the genes present in this list have been shown to have functional CSREs (15). Eleven genes that were reported to be *CAT8*-dependent (11) were not *CAT8*-dependent in our microarrays or did not appear in our data (Table IV, Supplemental Material, 3rd column). One of the former, *CAT2*, was just below the λ cutoff value for significance in our data.

Thus, there is good agreement between the miniarray/proteomics and microarray data considering that the previous genomic study assayed only half of the existing yeast ORFs using miniarrays. *ACS1*, previously known to be both *ADR1*- and *CAT8*-dependent, behaves as expected in both microarrays. In the single mutants, its *CAT8/cat8* and *ADR1/adr1* expression ratios are 3 and 9, respectively. In the double mutant, its *ADR1CAT8/adr1cat8* expression ratio is close to 10 (Tables I and IV of the Supplemental Material). Six of the *CAT8*-dependent genes in Table IV, Supplemental Material, are also *ADR1*-dependent (denoted with asterisks). The genes co-regulated by both *ADR1* and *CAT8* are not restricted to the pathway of ethanol oxidation (*ADH2*, *ACS1*). Lactate utilization (*JEN1*), meiosis (*ADY2*), and amino acid metabolism (*PUT4, ALP1*) are all potentially co-regulated by *CAT8* and *ADR1*. In the total set of 200 *CAT8*-dependent genes, 30 are *ADR1*-dependent. Because most of these genes lack recognizable CSREs, they may be affected indirectly by the absence of *CAT8*.

Confirmation of ADR1 Dependence of Gene Expression—The *ADR1* dependence of expression of several genes identified on the microarrays was confirmed by constructing promoter-*lacZ* gene fusions. The results in Fig. 5 confirm that expression of *ADY2*, *FDH2*, *ALD4*, and *YIL057C* is *ADR1*-dependent. The

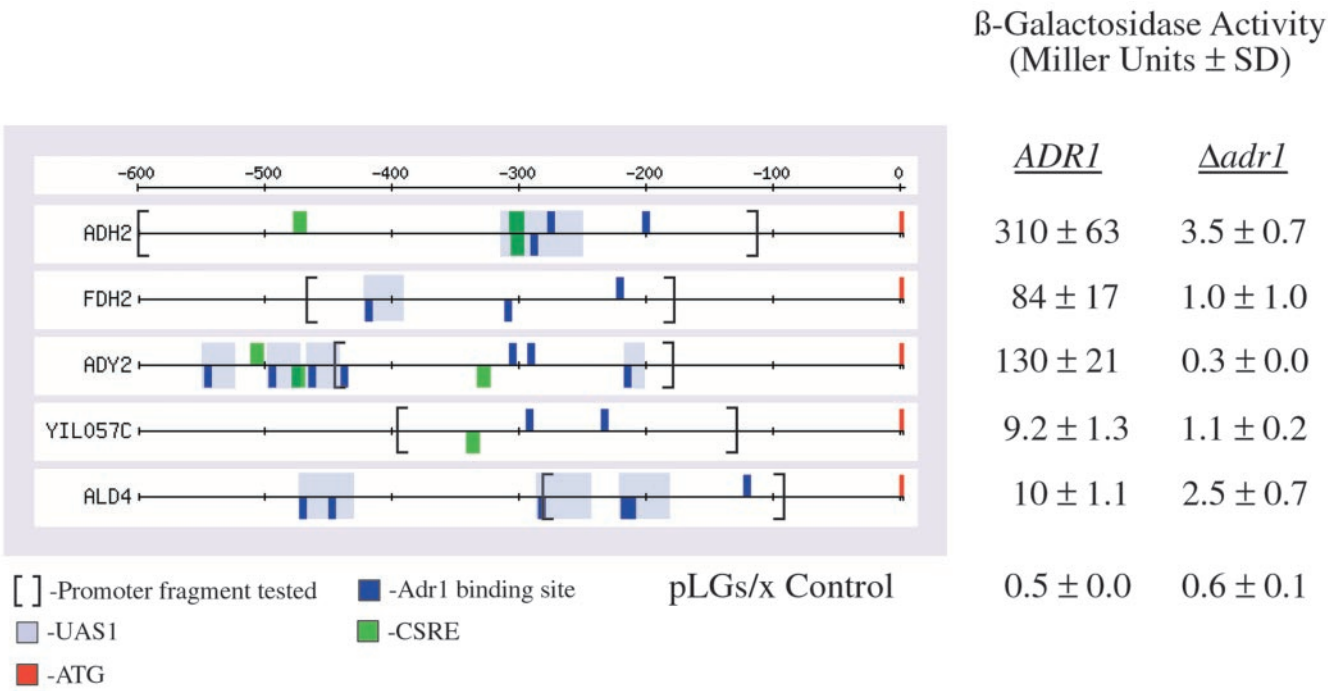

FIG. 5. **Confirmation of *ADR1* dependence using promoter-*lacZ* reporter genes.** Promoter-*lacZ* reporter plasmids were constructed from three independent PCR products for each promoter region to be tested. Yeast strains TYY320 (*ADR1*) and TYY324 (Δ*adr1*) were then transformed with each reporter plasmid. The promoter regions tested, numbered in base pairs relative to the ATG translational start site, are as follows: *ADH2*, −600 to −110 (54); *FDH2*, −468 to −182; *ADY2*, −445 to −187; *YIL057C*, −397 to −136; and *ALD4*, −280 to −91. These are demarcated on the transcription factor-binding site maps of each promoter by *brackets.* The pLGs/x control plasmid is the pLG669-Z plasmid lacking the UAS region of the *CYC1* promoter (54). Transformants were grown at 30 °C in ura$^-$ trp$^-$ selective medium containing 5% glucose to a cell density of about 2×10^7 cells/ml. The cells from each culture were collected by centrifugation and resuspended in selective medium containing 0.05% glucose. These cells were incubated at 30 °C for 6 h, the same length of time cultures used to prepare poly(A) RNA for microarray analyses were allowed to derepress. Whole cells were permeabilized, and β-galactosidase activity was measured as described under "Experimental Procedures." Each value represents the average β-galactosidase activity of the three independent promoter-*lacZ* plasmids constructed for each promoter.

ADR1 dependence of *IME1* expression was similarly confirmed. *IME1* is a master regulator of meiotic-specific gene expression. It has a large and complex upstream regulatory region that controls its activity in response to ploidy and growth conditions (53). Its known activity is important only in diploids undergoing meiosis. Because *ADR1*-dependent *IME1* expression in haploid cells is unexpected, we tested the activity of an *IME1/lacZ* gene fusion in *adr1/adr1*-homozygous diploids and *ADR1/adr1* heterozygous diploids, as well as in haploids. *IME1/lacZ* activity increased about 10-fold in diploids relative to haploids but still showed significant *ADR1* dependence (Table V). *adr1/adr1*-homozygous diploids are sporulation-competent, despite the fact that *IME1/lacZ* expression is reduced about 5-fold in *adr1-null* homozygous diploids.[3]

ADR1-dependent Genes Are Enriched in the Adr1 Consensus Binding Motif—ChIP experiments showed that Adr1 bound to the promoters of *ADR1*-dependent genes *ADH2*, *ACS1*, *CTA1*, *POT1*, and *GUT1* only in derepressed cells (20), demonstrating that Adr1 acts directly on these genes. To find additional direct targets of Adr1, we analyzed the promoters of the 50 genes having the highest degree of Adr1 dependence using an algorithm that searches DNA sequences for common motifs. The Adr1-binding consensus motif, (T/G)(T/C)GGRG, is recovered as the most frequent 6-bp sequence in the promoter region located within 1000 bp upstream of the ATG codon of these genes, indicating that *ADR1*-dependent genes are enriched in the Adr1 consensus binding motif. In contrast, the promoters of genes that appeared to be repressed by Adr1 have a TATA-like sequence as their most frequent motif.[4]

The promoters of all genes displaying *ADR1* dependence were also examined directly for the presence of Adr1-binding sites. Single Adr1-binding sites matching the consensus (31) perfectly were found in 90% of the promoters of the *ADR1*-dependent genes but in only 70% of the promoters in the total genome (Table V, Supplemental Material). In isolation, single Adr1-binding sites or direct repeats of a single site are not able to activate transcription (54). The activating sequence in the *ADH2* promoter, UAS1, consists of an inverted repeat of Adr1-binding sites. The UAS1 consensus sequence was present in 18% of the *ADR1*-dependent promoters as compared with only 4% of all promoters from the whole genome. Similarly, imperfect UAS1 consensus sequences were found in 70% of the *ADR1*-dependent promoters as compared with only 51% of promoters from the whole genome. Surprisingly, some of the *ADR1*-dependent genes contained only direct repeats of the Adr1-binding motif and not UAS1 consensus sequences. In particular, *YIL057C*, which is one of the most strongly *ADR1*-dependent genes, has two Adr1-binding sites in tandem orientation (Fig. 5). ChIP experiments demonstrate that Adr1 binds directly to the *YIL057C* promoter indicating that Adr1 can bind and activate through direct repeats (see below, Fig. 7).

Adr1-dependent Genes Have Promoters Enriched in OREs but Not CSREs—Because some *ADR1*-dependent genes are also dependent on either Cat8 or Oaf1/Pip2 for expression, we also searched for their binding sites, CSRE and ORE motifs, respectively. The promoters of *ADR1*-dependent genes are not enriched in CSRE sequences. Seventeen percent of both *ADR1*-dependent and total genomic promoter sequences have such sequences in their promoters (Table V, Supplemental Material). No discernible pattern of Adr1-binding sites or UAS1 positioning with respect to CSREs was observed. *ADR1*-dependent promoters, however, are enriched in ORE sequences. OREs

[4] V. Voronkova, personal communication.

TABLE V
ADR1-dependent expression of IME1/lacZ in haploid and diploid

Haploid strains TYY201 and TYY202, *ADR1* and *adr1Δ1::LEU2,* respectively, with pWL21 (*IME1/lacZ, URA3*) and diploid strains TGY25 and TGY27, *adr1Δ1::LEU2/adr1Δ1::LEU2* and *adr1Δ1::LEU2/ADR1,* respectively, with pWL21 (*IME1/lacZ, URA3*), were grown in selective media (ura$^-$) with 5% glucose (R) to about 2×10^7 cells/ml. A portion of the culture was assayed for β-galactosidase activity (R) as described under "Experimental Procedures," and cells from another portion were collected by centrifugation and resuspended in selective media plus 0.05% glucose (DR) or 0.1% potassium acetate (SPM) and grown for 18 h at 30 °C. A portion of each culture was removed and assayed for β-galactosidase activity (DR and SPM, respectively). The β-galactosidase activities are Miller units, and the standard deviations of the assays were about 20%.

Strain	Relevant genotype	Growth conditions		
		R	DR	SPM
TYY461	*ADR1*	0.04	1.0	ND
TYY462	*adr1Δ1::LEU2*	0.03	0.02	ND
TGY27	*adr1Δ1::LEU2/ADR1*	ND	7.7	3.8
TGY25	*adr1Δ1::LEU2/adr1Δ1::LEU*	ND	0.09	0.74

were found in 25% of all *ADR1*-dependent genes compared with 11% of all genes in the entire genome. Fourteen of the 27 *ADR1*-dependent genes containing OREs have UAS1-like sequences adjacent to an ORE (Fig. 6). Work by Gurvitz *et al.* (9) suggests that a cooperative interaction between the Oaf1-Pip2 complex and Adr1 may play a role in controlling β-oxidation and peroxisomal proliferation. Our findings support and extend their work.

Adr1 Binds to the Promoters of Many ADR1-dependent Genes—To determine whether Adr1 binds to the promoters of genes whose expression is *ADR1*-dependent in the microarray assay, a subset of the promoters was analyzed in ChIP experiments. As shown in Fig. 7, a hemagglutinin epitope-tagged version of Adr1 binds to the promoters of *ADY2*, *ALD4*, *POX1*, *CIT3*, *GIP2*, *ICL2*, *ETR1*, *YIL057C*, and *FDH2* in derepressed growth conditions.

ADR1-dependent Growth—Yeast strains lacking *ADR1* are unable to grow on glycerol or oleate as a sole carbon source (6, 49, 55), but our microarray data suggested that *ADR1* may influence growth on a wider range of carbon sources. Fig. 8 shows that *adr1* mutant cells grow poorly on medium containing glycerol, ethanol, citrate, or pyruvate as a carbon source. In addition, *adr1* mutant strains grow poorly on low glucose in the presence of formate, presumably because formate cannot be metabolized in the absence of *ADR1*-dependent expression of formate dehydrogenase. However, *adr1* mutant cells are able to grow on synthetic minimal medium containing the non-fermentable substrates acetate or lactate as the sole carbon source.

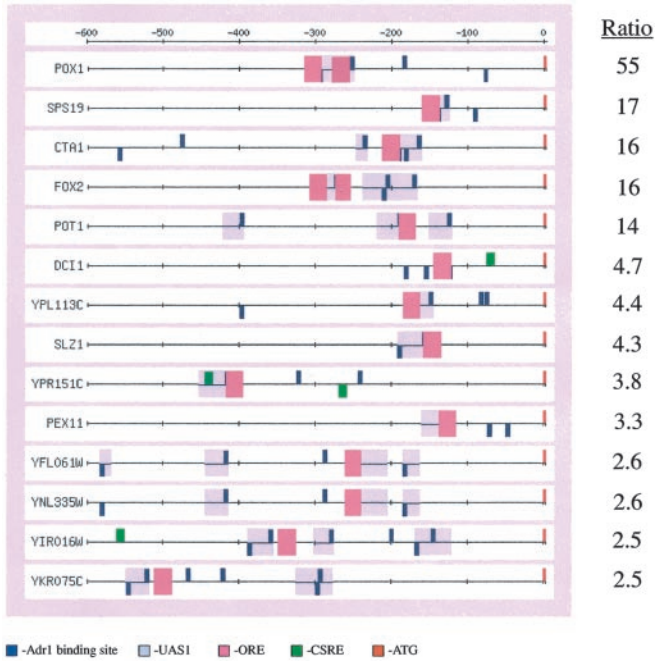

FIG. 6. ***ADR1*-dependent promoters that have adjacent Adr1-binding site or UAS1-ORE sequence elements.** Transcription factor-binding site maps were produced as described under "Experimental Procedures" and are listed in order of *ADR1* dependence. The value to the *right* of each map is the average expression ratio from the *ADR1*/Δ*adr1* microarray experiments.

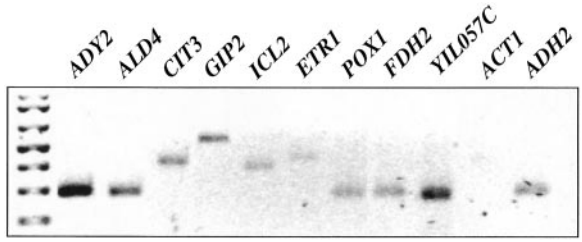

FIG. 7. **Adr1 protein binds to the promoters of *ADR1*-dependent genes.** Chromatin immunoprecipitation was used to enrich for Adr1-bound DNA from a strain with hemagglutinin-tagged Adr1 grown in depressing conditions as described in the legend to Fig. 5. Enriched DNA (1:1280 dilution) was used in PCRs with primer pairs for the promoters of genes (Table II) that were *ADR1*-dependent in the microarray experiments. Primers for *ACT1* and *ADH2* promoters were included as negative and positive controls, respectively.

DISCUSSION

Yeast growth in the absence of glucose relies on the breakdown of partially oxidized substrates that are derived from fermentative reactions. These metabolic products provide energy and intermediates for additional growth and cell division after glucose has been exhausted. The major source of metabolites and energy after the diauxic transition is the ethanol produced during fermentation.

Ethanol Metabolism—Genes encoding all of the enzymes required for the oxidation of ethanol, alcohol dehydrogenase, aldehyde dehydrogenase, and acetyl-CoA synthetase, show a high degree of *ADR1* dependence. This group includes *ADH2* and *ACS1*, two genes previously known to be *ADR1*-, *CAT8*-, and *SNF1*-dependent (3, 4, 11). Expression of *ADH3*, encoding a mitochondrial isozyme, and *ADH5*, encoding an alcohol dehydrogenase of unknown function, also appear to be *ADR1*-dependent. However, cDNAs derived from these two genes might co-hybridize with *ADH2* cDNA on the array because they are about 74% identical. Because *ADH2* is very highly expressed, if the cDNA derived from its abundant mRNA also hybridized to *ADH3* and *ADH5* on the array, they could be erroneously classified as *ADR1*-dependent. Two genes encoding aldehyde dehydrogenases, *ALD4* and *ALD5*, are *ADR1*-dependent. A third aldehyde dehydrogenase, encoded by *ALD6,* is *CAT8*-dependent for expression (Table IV) (11). These genes share only 60% identity and would not be expected to cross-hybridize significantly under the microarray conditions. Thus, the ethanol-oxidation pathway utilizes multiple isozymes, many of which are regulated by Adr1 and/or Cat8, to generate acetyl-

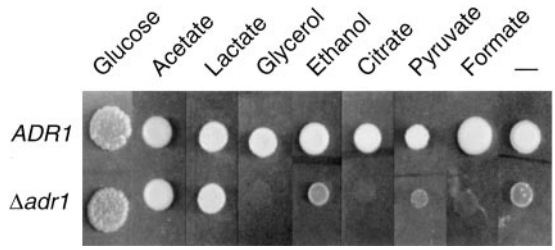

FIG. 8. ***ADR1* dependence of growth on different non-fermentable carbon sources.** Strains TYY201 (*ADR1*) and TYY202 (*adr1*) were grown on YPD plates overnight. Several colonies were picked into 1 ml of H_2O, and 5-fold serial dilutions were made and spotted onto plates containing synthetic medium with complete amino acid solution, uracil, adenine, and the indicated carbon sources. The concentrations of the carbon sources are indicated in *parentheses.* The spots shown contain about 1000 cells.

CoA during non-fermentative growth.

ADR1 and *CAT8* are required for ethanol oxidation to occur efficiently because *adr1* and *cat8* mutants grow poorly on ethanol as a sole carbon source. Use of acetate as a sole carbon source, on the other hand, requires *CAT8* but not *ADR1*. This observation suggests that at least one of the steps preceding acetate synthesis has an essential requirement for *ADR1*. Because ADHI should be able to substitute for ADHII in oxidizing ethanol, this result suggests that *ALD4* is the major acetaldehyde dehydrogenase that is most important for ethanol utilization. The requirement for *CAT8* for growth on both ethanol and acetate could reflect its importance for expression of *ACS1* and genes of the glyoxylate cycle and gluconeogenesis, which are needed to generate essential intermediates for cell growth.

Glycerol Metabolism—Growth on glycerol as a carbon source requires the activity of two enzymes. Glycerol kinase, encoded by *GUT1*, catalyzes the first step in glycerol breakdown. Glycerol-3-phosphate dehydrogenase, encoded by *GUT2*, is an enzyme of the inner membrane of the mitochondria whose major role in glycerol metabolism is in the glycerophosphate shuttle. This process transfers reducing equivalents across the NADH-impermeable mitochondrial inner membrane. Both genes are regulated by *INO2* and *INO4*, genes encoding transcription factors that are responsive to inositol signaling (56). Growth on glycerol is *ADR1*-dependent (6), presumably because of the dependence of *GUT1* and *GUT2* expression on *ADR1* (5, 6). In the DNA array experiments (Fig. 2 and Table III) both *GUT1* and *GUT2* are 2-fold *ADR1*-dependent for expression in low glucose-containing medium. It would be surprising if a 2-fold dependence on *ADR1* caused glycerol-deficient growth. Perhaps the full *ADR1* dependence of these genes is only evident in cells growing on glycerol, or maybe the level of their mRNAs is not predictive of Gut1 and Gut2 enzyme levels. Alternatively, other reactions that are compromised in the absence of Adr1 could contribute to the glycerol-deficient growth.

Lactate Metabolism—Lactate metabolism is a third pathway utilized by yeast in the absence of glucose. *Saccharomyces cerevisiae* contains three genes encoding enzymes capable of converting lactate to pyruvate. *CYB2* encodes an L-lactate dehydrogenase (cytochrome b_2) that is *ADR1*-dependent for expression, confirming previous suggestions that *ADR1* might play a role in *CYB2* derepression (57). *DLD1*, encoding a D-lactate dehydrogenase isozyme, is *CAT8*-dependent, as is a lactate symporter encoded by *JEN1* (11). Expression of *JEN1* may also be *ADR1*-dependent. However, the significance of its expression ratio is unclear because it has an associated λ value just below the established cutoff. A third lactate dehydrogenase is encoded by *DLD3*, expression of which is elevated in the absence of Adr1 (Table I, Supplemental Material). ORF YPL113C encodes a lactate dehydrogenase homolog of unknown function. This ORF is *ADR1*-dependent for expression. Thus, lactate metabolism, as with ethanol metabolism, utilizes multiple isozymes that are differentially regulated by *ADR1* and *CAT8*. *ADR1* appears to regulate genes involved in L-lactate utilization, whereas *CAT8* appears to regulate D-lactate utilization.

The ability of *adr1* mutants to grow on lactate is surprising because several genes in the pathway depend on *ADR1* for expression. Lactate is metabolized to acetyl-CoA via pyruvate, and *adr1* mutant cells cannot grow on pyruvate. An explanation for this apparent conundrum might be that while pyruvate can be utilized by *adr1* mutants if it is generated internally, perhaps it cannot be transported into the cell, or into the mitochondria, in the absence of carriers that are dependent on *ADR1* for expression. This scenario might also explain the inability of *adr1* mutants to grow on citrate. This explanation seems reasonable because several mitochondrial carboxylate carriers, such as Ctp1, Dic1, and Oac1, are expressed in an *ADR1*-dependent fashion.

Formate Metabolism—Two genes encoding formate dehydrogenases *FDH1* and *FDH2* are among the most highly *ADR1*-dependent genes in yeast. These two recently diverged ORFs are 94% identical and are not distinguishable by the array hybridization conditions. In some strains, including W303 studied here, *FDH2* is inactive due to a frameshift mutation (36). The primary role of formate dehydrogenase in yeast metabolism is unknown, but formate can be co-metabolized with growth-limiting amounts of glucose to increase the yield of yeast biomass. This is thought to result from oxidation of formate to CO_2, which increases the amount of cytosolic NADH available for the production of ATP (36). A putative formate/nitrite transporter encoded by YHL008C (Table III) is also expressed in an *ADR1*-dependent manner.

Genes Encoding Mitochondrial Transport Proteins—ADR1 is involved in the expression of genes encoding mitochondrial inner membrane proteins that serve as transporters of intermediates in tricarboxylic acid and glyoxylate cycles. *CTP1*, encoding a citrate transport protein, and *OAC1*, encoding a transporter of oxaloacetate, are both *ADR1*-dependent for expression (Table III). Citrate transport is important because it allows citrate, formed from the reaction of acetyl-CoA and oxaloacetate in the mitochondrion, to be transported to the cytoplasm where it can be utilized for fatty acid biosynthesis and gluconeogenesis after being broken down to acetyl-CoA and oxaloacetate. This is the only mechanism known that allows acetyl-CoA produced in the mitochondria to be utilized in other cellular compartments. *DIC1*, encoding a dicarboxylic acid transporter in the mitochondrial inner membrane, is also expressed in an *ADR1*-dependent fashion.

β-Oxidation—The utilization of exogenous fatty acids for yeast growth is dependent on *ADR1*, *OAF1*, and *PIP2* (8, 9, 32). *OAF1* is expressed constitutively, whereas expression of *PIP2* is regulated (58). The heterodimeric Oaf1-Pip2 transcription factor is active only in the presence of an inducing fatty acid such as oleate. Thus, in the absence of oleate Adr1 appears to be sufficient to derepress all of the genes necessary for the β-oxidation of fatty acids (Table III). However, some genes needed for use of exogenous fatty acids must require Oaf1-Pip2 for their expression because *OAF1* and *PIP2* are needed for growth on oleate. Genes that encode proteins that transport fatty acids across the cell and peroxisomal membranes are likely candidates, but the hypothesized transporter genes have not been identified.

A Methyl Citrate Cycle and Propionate Metabolism in S. cerevisiae—The β-oxidation pathway can metabolize even- or odd-numbered fatty acids. However, if the acyl-CoA sub-

strate contains an odd number of carbon atoms, propionyl-CoA rather than acetyl-CoA is the product of the last cycle of the reaction. Propionate is also derived from amino acid catabolism and can be converted to propionyl-CoA by acetyl-CoA synthase. Propionyl-CoA and oxaloacetate form 2-methyl citrate which is metabolized via the methyl citrate cycle to pyruvate and succinate (Fig. 2), thus generating pyruvate for mitochondrial oxidation and replenishing the tricarboxylic acid cycle with succinate. Based on their coordinate induction in cells with dysfunctional mitochondria, it was proposed that enzymes encoded by *CIT3*, *ACO1*, and *ICL2* (59) catalyze the methyl citrate cycle in *S. cerevisiae* (60). *PDH1*, another co-induced gene, was hypothesized to carry out an essential function in propionate metabolism in *S. cerevisiae* because its deletion rendered cells sensitive to exogenous propionate.

CIT3 and *ICL2* are highly *ADR1*-dependent suggesting that *ADR1* might be involved in the response to dysfunctional mitochondria. *PDH1* is a homolog of a bacterial dehydratase that preferentially utilizes 2-methyl citrate, rather than citrate, in the first step of the aconitase reaction, forming 2-methyl *cis*-aconitate (61, 62). All three genes are also highly glucose-repressed (Table IV). It is possible that *CIT3*, *PDH1*, and *ICL2* perform the three steps of the methyl citrate cycle of propionate metabolism, rather than *CIT3*, *ACO1*, and *ICL2*, as suggested previously, and that the expression of the genes encoding the enzymes of the entire pathway is *ADR1*-dependent. If *PDH1* is a methylcitrate dehydratase but not an aconitase, a fourth enzyme would be needed to form methyl isocitrate from 2-methyl *cis*-aconitate. Aconitase could carry out this function, or there could be an unidentified ORF encoding an enzyme that preferentially hydrates 2-methyl *cis*-aconitate. *YJL200C* encodes a protein with significant homology to *ACO1* and could be the unidentified hydratase. As described below, the expression of *BAT1*, encoding a branched amino acid aminotransferase that makes propionate from isoleucine, is also *ADR1*-dependent, thus forging another link between *ADR1* and the putative methyl citrate cycle.

ADR1 and Retrograde Regulation—Our data also suggest a connection between *ADR1* and the sensing of mitochondrial function. Butow and colleagues (60) have characterized a yeast pathway called "retrograde regulation" that alters nuclear gene expression in response to changes in mitochondrial function. The genes that are regulated by this pathway and the genes that are regulated by Adr1 show considerable overlap. DNA microarray analysis identified 43 genes that are at least 2-fold induced in cells lacking mitochondrial DNA. Thirty five of these genes were present on our microarrays, and 17 of them are *ADR1*-dependent for expression. Thus, it is possible that *ADR1* is involved in intracellular communication between the mitochondria and nucleus.

ADR1-dependent Promoters—Analysis of the promoters of many of the *ADR1*-dependent genes indicates that they contain putative binding sites for Adr1, suggesting that Adr1 could have a direct effect on the expression of these genes. Adr1 binding was demonstrated for an additional 9 genes, bringing to 14 the number of promoters known to bind Adr1 *in vivo*. Many of the genes of the β-oxidation pathway contain binding sites for both Adr1 and Oaf1.Pip2, providing further support for the idea that these two transcription factors coordinately regulate the transcription of these genes in a direct manner.

SNF1-dependent Gene Expression—As expected *SNF1* plays a major role in gene expression in the absence of glucose. Among the genes dependent on *SNF1* for transcription are transcription factors and other regulatory proteins. Many of the effects of *SNF1* on transcription could act through these genes (Table III, Supplemental Material). For example, *CAT8* and *SIP4* are two transcription factors that are known to be dependent on *SNF1* for expression and activity (10, 15, 18). *CAT8* expression is *SNF1*-dependent and glucose-repressed in our microarray data, as is expression of its functional homolog *SIP4*. This could explain the dependence of the genes encoding glyoxylate cycle and gluconeogenic enzymes on *SNF1* for derepression (Table I, Supplemental Material).

SNF1-dependent expression of the transcription factors encoded by *ZAP1* and *CUP9* may explain the dependence of some of the metal- and peptide-transport proteins on *SNF1* (Table II, Supplemental Material). *CUP9*-dependent effects on copper ion homeostasis are most pronounced in the absence of glucose (63), consistent with *SNF1*-dependent *CUP9* expression. *SNF1*-dependent expression of sporulation-specific proteins may be transmitted through the transcription factor *IME1*, the early meiotic regulator.

Two ORFs encoding putative transcription factors are also expressed in a *SNF1*-dependent manner, *YOX1* and YGR067C (Table III, Supplemental Material). YGR067C encodes a C2H2 zinc finger protein whose predicted DNA recognition properties are identical to those of *ADR1*, based on the amino acid sequence of its two zinc fingers. It is 30-fold derepressed in a *SNF1*-dependent manner, and its expression is also dependent on *CAT8* (10) (see Table I, Supplemental Material). It could activate or repress many of the same genes that are regulated by *ADR1* and be part of a feedback loop linking UAS1-containing genes to *SNF1* and *CAT8*.

SNF1 controls expression of several genes encoding protein phosphatase and protein kinase regulatory subunits (Table III, Supplemental Material). *REG2*, a homolog of the type I protein phosphatase regulatory subunit encoded by *REG1,* is strongly derepressed in a *SNF1*-dependent fashion, as is another gene encoding a phosphatase-regulatory subunit, *GAC1.* A subunit of the Snf1 kinase complex, Sip2, is derepressed in an *SNF1*-dependent manner, suggesting a positive transcription feedback loop. Several other protein kinase regulatory subunits are also expressed in an *SNF1*-dependent fashion, including the two mitotic cyclins, *CLB1* and *CLB2*. Thus, *SNF1* has the potential for affecting transcription more globally by enhancing expression of upstream regulatory factors in addition to acting directly on specific transcription factors.

SNF1 does not seem to be important for expression of any components of the polII holoenzyme complex (Table I, Supplemental Material). No genes encoding polII subunits, general transcription factors, subunits of the mediator complex, or components of chromatin-modifying complexes are *SNF1*-dependent for expression. Thus, *SNF1* must affect transcription in derepressed cells by altering the activity and expression of specific regulatory proteins or by altering the activity of the holoenzyme.

Our data are consistent with the regulatory network model of gene expression suggested by the recent genome-wide location analysis (64). Adr1, Cat8, and Snf1 play important roles in the expression of other transcriptional regulatory genes, parlaying an influence on direct target genes into a more global effect on genome expression. Equally important is the potential interaction between Adr1 and other transcription factors on the promoters of *ADR1*-dependent genes. These two aspects of Adr1 function contribute to its broad influence on gene regulation after glucose depletion. Adr1 is a key coordinator of pathways that metabolize non-fermentable carbon sources, allowing yeast to respond effectively to changes in nutrient conditions.

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