MOLECULAR AND CELLULAR BIOLOGY, Apr. 1995, p. 1915–1922 Vol. 15, No. 4
0270-7306/95/$04.00+0

CAT8, a New Zinc Cluster-Encoding Gene Necessary for Derepression of Gluconeogenic Enzymes in the Yeast *Saccharomyces cerevisiae*

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Received 21 November 1994/Returned for modification 20 December 1994/Accepted 6 January 1995

The expression of gluconeogenic fructose-1,6-bisphosphatase (encoded by the *FBP1* gene) depends on the carbon source. Analysis of the *FBP1* promoter revealed two upstream activating elements, $UAS1_{FBP1}$ and $UAS2_{FBP1}$, which confer carbon source-dependent regulation on a heterologous reporter gene. On glucose media neither element was activated, whereas after transfer to ethanol a 100-fold derepression was observed. This gene activation depended on the previously identified derepression genes *CAT1* (*SNF1*) (encoding a protein kinase) and *CAT3* (*SNF4*) (probably encoding a subunit of Cat1p [Snf1p]). Screening for mutations specifically involved in $UAS1_{FBP1}$ derepression revealed the new recessive derepression mutation *cat8*. The *cat8* mutants also failed to derepress $UAS2_{FBP1}$, and these mutants were unable to grow on nonfermentable carbon sources. The *CAT8* gene encodes a zinc cluster protein related to *Saccharomyces cerevisiae* Gal4p. Deletion of *CAT8* caused a defect in glucose derepression which affected all key gluconeogenic enzymes. Derepression of glucose-repressible invertase and maltase was still normally regulated. A *CAT8-lacZ* promoter fusion revealed that the *CAT8* gene itself is repressed by Cat4p (Mig1p). These results suggest that gluconeogenic genes are derepressed upon binding of Cat8p, whose synthesis depends on the release of Cat4p (Mig1p) from the *CAT8* promoter. However, gluconeogenic promoters are still glucose repressed in *cat4* mutants, which indicates that in addition to its transcription, the Cat8p protein needs further activation. The observation that multicopy expression of *CAT8* reverses the inability of *cat1* and *cat3* mutants to grow on ethanol indicates that Cat8p might be the substrate of the Cat1p/Cat3p protein kinase.

Growth of *Saccharomyces cerevisiae* on nonfermentable carbon sources such as ethanol, lactate, or glycerol requires the enzymes of both the glyoxylate cycle and the gluconeogenic pathway. Fructose-1,6-bisphosphatase is a key enzyme in gluconeogenesis. The specific activity and the amount of this enzyme in yeast cells are subject to strict regulation depending on the availability of glucose in the growth medium (reviewed in references 10, 16, 24, and 51). Several regulatory mechanisms have been described for fructose-1,6-bisphosphatase: (i) allosteric inhibition by AMP and fructose-2,6-bisphosphate (15, 28), (ii) reversible inactivation by phosphorylation (interconversion) (29, 32), (iii) proteolytic degradation by glucose inactivation (14), and (iv) repression of transcription by glucose (glucose repression) (15, 20, 46).

Regulation by glucose repression is an important mechanism for long-term adaptation to glucose as a carbon substrate. Although several glucose repression mutants have been isolated and characterized, none of these mutations revealed a defect in glucose repression of gluconeogenic enzymes (for reviews, see references 9 and 16). Only mutations that affect derepression after release from glucose repression have been found. The derepression genes *CAT1* (*SNF1* or *CCR1*) (2, 6, 55) and *CAT3* (*SNF4*) (2, 11) are central regulatory elements. *CAT1* (*SNF1*) encodes a protein-serine/threonine kinase (3) with the *CAT3* (*SNF4*) product as a subunit necessary for its function (4, 12).

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The two kinds of mutants obtained so far indicate positive derepression and negative repression regulatory systems. The positive derepression system affects all known glucose-repressible genes, whereas mutations in the negative repression system do not interfere with gluconeogenic enzymes.

To understand the molecular basis of glucose derepression, the *FBP1* promoter was carefully analyzed, and two upstream activation sites (UASs), $UAS1_{FBP1}$ and $UAS2_{FBP1}$, involved in the derepression regulatory system were identified. Deletion of both UAS elements together abolished *FBP1* expression, and gel retardation experiments revealed carbon source-dependent protein complexes for both elements (37). To confirm the importance of these *cis*-acting derepression elements, we examined their function in a heterologous reporter gene construct. To identify *trans*-acting elements which specifically regulate $UAS1_{FBP1}$ derepression, we used the $UAS1_{FBP1}$-*CYC1-lacZ* fusion to isolate $UAS1_{FBP1}$ derepression mutants. We could identify a new derepression gene, designated *CAT8*, whose characterization is described in the present report.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used are listed in Table 1. Strain WAY.5-4A is the wild-type strain used to isolate the mutants described in this study. Recombinant plasmids were amplified in *Escherichia coli* DH5α [F^- ϕ80d/*lacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*].

The compositions of yeast rich medium (yeast extract-peptone based) and synthetic complete (SC) medium have been described previously (36). Yeast synthetic complete and rich media contained 4% glucose, 4% raffinose, 4% maltose, or 3% ethanol as a carbon source.

For screening of mutants, derepression conditions were achieved by growth in SC medium plus 0.2% glucose and 3% ethanol ($SCD_{0.2}E_3$ medium). For detection of β-galactosidase on plates, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype[a]
WAY.5-4A	*MAT***a** *ura3-52 his3-Δ1 MAL2-8^c MAL3 SUC3*
JS87.11-7C	*MATα ura3-52 leu2-3,112 cat1::HIS3 MAL2-8^c MAL3 SUC3*
JS87.15-7D	*MAT***a** *ura3-52 trp1-284 cat3::LEU2 MAL2-8^c MAL3 SUC3*
JS88.3-1A	*MAT***a** *ura3-52 his3-Δ1 cat4-1 MAL2-8^c MAL3 SUC3*
PK18/1	*MAT***a** *ura3-52 cat4::HIS3 MAL2-8^c MAL3 SUC3*
ENY.cyc8-7B	*MAT***a** *ura3-52 leu2-3,112 his3-Δ1 cyc8-20 MAL2-8^c MAL3 SUC3*
WAY.6-2B	*MATα ura3-52 hxk2::LEU2 MAL2-8^c MAL3 SUC3*
WAY.5-4A/65	*MAT***a** *ura3-52 his3-Δ1 cat8-65 MAL2-8^c MAL3 SUC3*
WAY.5-4A/160	*MAT***a** *ura3-52 his3-Δ1 cat8-160 MAL2-8^c MAL3 SUC3*
DG2	*MAT***a** *ura3-52 cat8::HIS3 MAL2-8^c MAL3 SUC3*
DG60	*MAT***a** *ura3-52 his3*::ΔUAS-*CYC1-lacZ*::*HIS3 MAL2-8^c MAL3 SUC3*
DG62	*MAT***a** *ura3-52 his3*::ODG13/14$_N$-*CYC1-lacZ*::*HIS3 MAL2-8^c MAL3 SUC3*

[a] *MATα* and *MAT***a** refer to mating types; *his3-Δ1*, *leu2-3,112*, *trp1-284*, and *ura3-52* cause nutritional requirements for amino acids and uracil; *MAL2-8^c* causes largely constitutive but still glucose-repressible synthesis of maltase (54); *MAL3*, another structural gene for maltase, is closely linked to *SUC3*, the structural gene for invertase (18).

β-D-galactoside) per ml was added to SC medium, which was buffered to pH 6.8 with 0.1 M potassium phosphate buffer.

E. coli strains were grown in Luria-Bertani medium (Gibco). For selection of antibiotic-resistant *E. coli* transformants, ampicillin (40 μg/ml) was added.

Synthetic oligonucleotides used for insertion studies. Oligonucleotides were synthesized on a 391 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany). For all sequences, complementary strands were designed to give ends compatible with an *Xho*I site. Double-stranded oligonucleotides ODG13/14 ($UAS1_{FBP1}$) (nucleotides −440 to −416 from *FBP1*, 5′-TCGAGGTCGTGCGGACACCCGGGAGTTATGAGATCT-3′) and ODG27/28 ($UAS2_{FBP1}$) (nucleotides −516 to −490 from *FBP1*, 5′-TCGAGCTCTCTTTTCCGGACGGATGGAATCGAT-3′) contained the UAS_{FBP1} elements (underlined) and additional sequences for cloning purposes.

Plasmid constructs. To characterize *cis*-acting regulatory sequences, plasmid pJS205 was used (45). Synthetic oligonucleotides or PCR fragments were inserted into the single *Xho*I site of pJS205. The orientations of inserts were determined by DNA sequencing. Plasmids pDG24 and pDG46 contain single insertions of oligonucleotides ODG13/14 and ODG27/28, respectively. Constructions pDG51N and pDG51R carry PCR fragment insertions including both UAS elements of *FBP1* (nucleotides −517 to −414). Episomal plasmids pJS205 and pDG24 were converted into the respective integrative plasmids pDG32 (ΔUAS-*CYC1-lacZ*) and pDG34 [$(ODG13/14)_N$-*CYC1-lacZ*] (N indicates normal orientation) by transfer of 4.3-kb *Sal*I-*Stu*I fragments containing the reporter gene cassettes into pRS303 (49) cleaved by the appropriate enzymes. Plasmid pJS234 (45) was used as a control for gene expression. In addition to plasmid pJS205, this plasmid contains a unique binding sequence of the general yeast activator protein Rap1 (47). To characterize *trans*-acting regulatory elements, plasmid pJS151 (*FBP1-lacZ URA3* 2μm) (37), which contains approximately 2.3 kb of the *FBP1* upstream region together with the first 19 codons of *FBP1* fused to the β-galactosidase gene, was used. Plasmid pCAT8 contains the *CAT8* gene cloned from an *S. cerevisiae* genomic DNA library in YCp50. Plasmids pDG161 and pDG166 are "dropout" derivatives of pCAT8. Plasmids pDG168, pDG169, pDG173, and pDG182 were generated by subcloning of distinct fragments from pCAT8 into YCplac33 (17). The structures of these plasmids are shown in Fig. 1. A *CAT8-lacZ* fusion was obtained by ligation of a 2-kb *Hin*dIII DNA fragment of the *CAT8* gene with *lacZ* fusion plasmid YEp356 (33). The resulting plasmid,

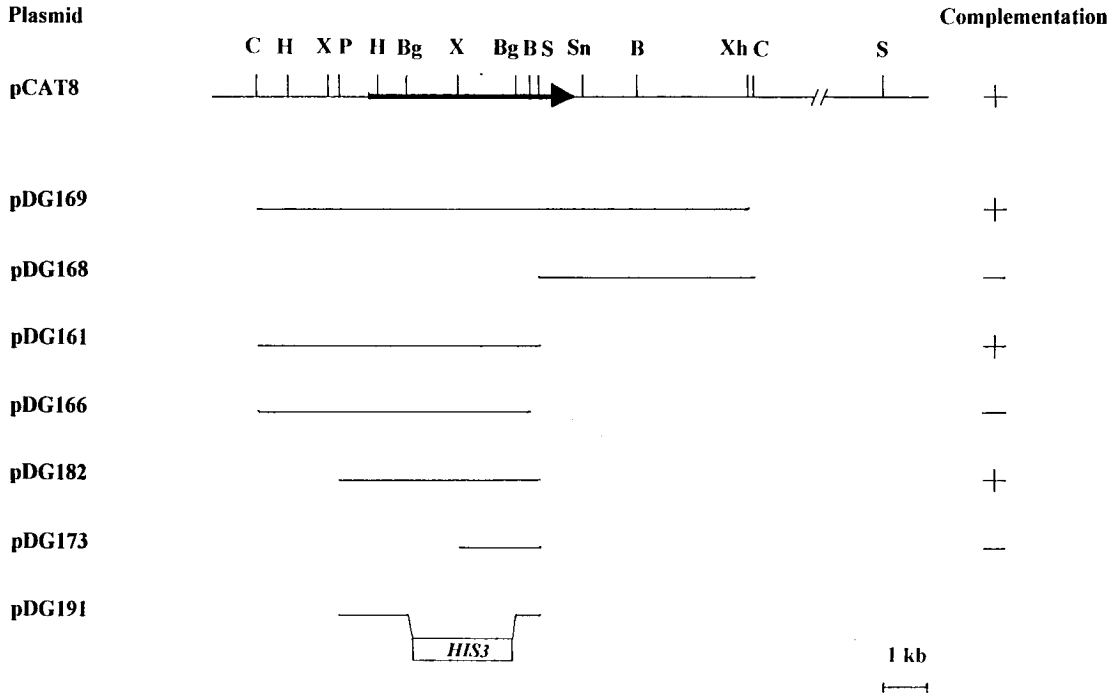

FIG. 1. Restriction map of *CAT8* gene and plasmids. Plasmids for complementation analysis are described in Materials and Methods. The arrow indicates the extent and 5′-to-3′ direction of the *CAT8* open reading frame. Results of growth complementation studies are summarized on the right (+, complementation; −, no complementation). The *Cla*I-*Sal*I fragment is 9.5 kb. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sn, *Sna*BI; X, *Xba*I; Xh, *Xho*I. Not all *Bgl*II, *Hin*dIII, *Sna*BI, and *Xba*I sites are shown. The *cat8*::*HIS3* gene disruption is shown in the bottom line (pDG191).

pCAT8lacZ, contains 1.8 kb of the *CAT8* promoter together with the first 69 codons of *CAT8* fused in frame to the β-galactosidase gene.

Mutagenesis and isolation of mutants. Yeast strain WAY.5-4A containing plasmid pDG24 was grown in SCD-uracil medium to the mid-log phase. For isolation of $UAS1_{FBP1}$ derepression mutants, cells were mutagenized with 1% ethyl methanesulfonate in water for 30 min (survival rate, about 50%) and washed twice with sterile water. After 6 h of incubation in SCD-uracil medium for mutation fixation, cells were washed and plated on $SCD_{0.2}E_3$ plates containing X-Gal (about 300 colonies per plate). White colonies were collected and rescreened. To exclude mutagenic effects on plasmid pDG24, this plasmid was removed from the putative mutants by nonselective growth on yeast extract-peptone-dextrose. Thereafter cells were retransformed with (nonmutagenized) pDG24 and in parallel with a Rap1-*CYC1-lacZ* fusion plasmid, pJS234. Finally, the putative mutants were tested for β-galactosidase activity. If the β-galactosidase activity was affected in pDG24 transformants but not in pJS234 transformants under derepression conditions, the respective mutants were further investigated.

Construction of a *cat8*::*HIS3* null allele. A 4.4-kb *Pst*I-*Sal*I fragment carrying the *CAT8* gene was subcloned into vector pBluescript II SK− (Stratagene), yielding pDG178. A 1.75-kb *Bam*HI fragment containing the *HIS3* gene was inserted into *Bgl*II-cleaved vector pDG178 to obtain pDG191. Prior to yeast transformation of strain WAY.5-4A, the *cat8*::*HIS3* insertion construction pDG191 was released by *Bam*HI digestion. Disruption of the chromosomal locus was verified by Southern blot analysis.

DNA sequencing. Restriction fragments were cloned in vector pBluescript II SK−, and the sequence was determined by the method of Sanger et al. (41) with reverse and M13-20 primers by using DNA sequencer 373 (Applied Biosystems). The sequence of other parts of the *CAT8* locus was determined by synthetic oligonucleotide-primed sequencing. The program TFASTA (38) was used to compare sequences with those in the GenBank database.

Recombinant DNA procedures. For standard recombinant DNA techniques, established protocols were followed (1, 40). Yeast transformation was performed as described by Klebe et al. (26). Isolation of plasmid DNA from yeast cells was performed as described by Hoffman and Winston (22).

Enzyme assays. Crude extracts were prepared with glass beads (5), and protein was determined by the microbiuret method (53) at 290 nm with bovine serum albumin as a standard. Invertase and maltase were assayed as described previously (36). β-Galactosidase was measured as described by Guarente (19). Specific β-galactosidase activities given in the tables are mean values from at least four independent measurements. Fructose-1,6-bisphosphatase activity was measured by the method described by Gancedo (14), phosphoenolpyruvate carboxykinase activity was measured as described by Hansen et al. (21), and isocitrate lyase activity was determined by the protocol described by Dixon and Kornberg (7).

SDS-PAGE and immunoblot analysis. Protein samples were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 6% resolving gel (27). Western transfer of proteins to a nitrocellulose filter was carried out as described by Towbin et al. (50). The filter was incubated with a mouse monoclonal anti-β-galactosidase antibody, washed, and subsequently treated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody. Bound antibody was visualized by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$).

Nucleotide sequence accession number. The GenBank/EMBL accession number for the *CAT8* sequence reported in this paper is X78344.

RESULTS

Functional analysis of $UAS1_{FBP1}$ and $UAS2_{FBP1}$ upstream regions. By deletion analysis we previously identified two UAS elements which are required for derepression of the *FBP1* gene (37). To verify the roles of $UAS1_{FBP1}$ and $UAS2_{FBP1}$ in glucose derepression, oligonucleotide fragments representing the core sequences of the supposed derepression activation elements $UAS1_{FBP1}$ (ODG13/14) and $UAS2_{FBP1}$ (ODG27/28) were inserted into the promoter test plasmid pJS205 (45). This high-copy-number plasmid without UAS insertion allows only low-level TATA-mediated gene expression of the *CYC1-lacZ* reporter gene. After oligonucleotide insertion, a significant stimulation of this basal gene expression, which depended on the carbon source provided, was observed (Table 2). On glucose one copy of $UAS1_{FBP1}$ increased the β-galactosidase activity about 1.5-fold. Upon derepression on ethanol, this activity increased a further 26-fold. The $UAS2_{FBP1}$ element conferred a 2.4-fold activation effect on glucose and a further 34-fold increase after derepression. The promoter test plasmid pJS205 showed a fourfold increase upon derepression, but this basal activation did not diminish the clear activating characteristics of both UAS elements. To investigate interactive effects of $UAS1_{FBP1}$ and $UAS2_{FBP1}$, a shortened 104-bp *FBP1* fragment (nucleotides −517 to −414) which contains both *cis*-regulatory elements and the 49-bp spacing region was inserted into the heterologous *CYC1-lacZ* construct. As shown in Table 2, insertion of both UASs increased the glucose-repressed and -derepressed activities additively. The activation did not depend on the orientation of the respective fragment (plasmids pDG51N and pDG51R in Table 2). Similar results were obtained by using episomal plasmids or integrative constructs targeted to the *HIS3* locus (plasmid pDG34 in Table 2).

TABLE 2. Insertion of synthetic oligonucleotides into a UAS test plasmid

Plasmid[a]	Construct[b]	Sp act of β-galactosidase (nmol/min/mg) with:	
		Glucose[c] (activation factor[d])	Ethanol[e] (derepression factor[d])
pJS205	ΔUAS-*CYC1-lacZ*	46 (1)	166 (4)
pDG24	$UAS1_N$-*CYC1-lacZ*	71 (1.5)	1,842 (26)
pDG46	$UAS2_N$-*CYC1-lacZ*	109 (2.4)	3,659 (34)
pDG51N	$UAS1+UAS2_N$-*CYC1-lacZ*	168 (3.7)	5,463 (33)
pDG51R	$UAS1+UAS2_R$-*CYC1-lacZ*	189 (4.1)	6,152 (33)
pDG32[f]	ΔUAS-*CYC1-lacZ*	6 (1)	11 (2)
pDG34[f]	$UAS1_N$-*CYC1-lacZ*	6 (1)	89 (15)

[a] Plasmids were transformed into wild-type strain WAY.5-4A.
[b] Subscript N or R indicates normal or reverse orientation, respectively, of the oligonucleotide or PCR fragment insert.
[c] Repressed growth conditions of wild-type transformants (SCD_4 medium lacking uracil).
[d] Ratio of β-galactosidase specific activities in transformants with respect to the ΔUAS reference (pJS205 or pDG32).
[e] Derepressed growth conditions of wild-type transformants (SCE_3 medium lacking uracil).
[f] Integrative plasmid targeted to the *HIS3* locus of wild-type strain WAY.5-4A. The resulting strains were designated DG60 (transformed with pDG32) and DG62 (transformed with pDG34).

Influence of *trans*-acting factors on *FBP1* gene activation. By using an *FBP1* promoter–β-galactosidase gene fusion construct (pJS151) (37), the function of $UAS1_{FBP1}$ and $UAS2_{FBP1}$ was examined in several mutants with defects in glucose repression and glucose derepression. The *cat1* (*snf1* or *ccr1*) and *cat3* (*snf4*) derepression mutants could not derepress the *FBP1* promoter–β-galactosidase gene fusion, indicating that *CAT1* (*SNF1* or *CCR1*) and *CAT3* (*SNF4*) are essential for its derepression (Table 3). In contrast, mutations in those genes nec-

TABLE 3. Influence of regulatory mutations on expression of a *FBP1-lacZ* reporter gene

Strain[a]	Relevant genotype	Sp act of β-galactosidase (nmol/min/mg) with:	
		Glucose[b]	Ethanol[c]
WAY.5-4A	Wild type	25	2,560
JS87.11-7C	*cat1*	32	33
JS87.15-7D	*cat3*	30	32
JS88.3-1A	*cat4*	20	2,150
ENY.cyc8-7B	*cyc8*	28	1,930
WAY.6-2B	*hxk2*	26	2,880

[a] The episomal reporter plasmid pJS151 (*FBP1-lacZ URA3* 2 μm) was used for transformation.
[b] Repressed growth conditions (SCD_4 medium lacking uracil).
[c] Derepressed growth conditions (SCE_3 medium lacking uracil).

TABLE 4. $UAS1_{FBP1}$-dependent gene activation in regulatory mutants

Strain	Relevant genotype	Sp act of β-galactosidase (nmol/min/mg) with:			
		Glucose[a]		Ethanol[b]	
		pJS205[c]	pDG24[d]	pJS205	pDG24
WAY.5-4A	Wild type	46	71	166	1,842
JS87.11-7C	*cat1*	34	101	121	132
JS87.15-7D	*cat3*	80	125	192	151
JS88.3-1A	*cat4*	80	106	132	1,560
ENY.cyc8-7B	*cyc8*	40	130	100	1,170
WAY.6-2B	*hxk2*	30	70	160	1,790

[a] Growth in SCD_4 medium lacking uracil.
[b] Growth in SCE_3 medium lacking uracil.
[c] ΔUAS-*CYC1-lacZ* reporter gene.
[d] $UAS1_N$-*CYC1-lacZ* reporter gene.

essary for glucose repression, such as *HXK2* (encodes hexokinase isoenzyme PII [8, 13]), *CYC8* (*SSN6*) (encodes a nuclear phosphoprotein containing 10 copies of a tetratricopeptide repeat motif [48]), and *MIG1* (*CAT4*) (encodes a zinc finger repressor protein [34, 35, 44]) had no influence on the regulation of the *FBP1* promoter–β-galactosidase gene fusion. Similar results, i.e., no effect on the regulation of the *FBP1-lacZ* fusion, could be obtained with the well-known glucose repression mutations *hex2* (*reg1*) and *cat80* (*grr1*) (data not shown).

In addition to the influence of regulatory factors on the complex *FBP1* promoter, we examined the effects of these genes on $UAS1_{FBP1}$-controlled synthetic promoters. The expression of reporter genes in plasmid pJS205 (ΔUAS-*CYC1-lacZ*) and plasmid pDG24 (UAS1-*CYC1-lacZ*) was measured in regulatory mutants under repressing or derepressing conditions. As shown in Table 4, the $UAS1_{FBP1}$-dependent gene activation was completely abolished in either the *cat1* or *cat3* mutant, indicating a positive regulatory effect of the respective proteins on $UAS1_{FBP1}$ derepression. Similar results were obtained for the $UAS2_{FBP1}$ element (data not shown). No *trans* influence was observed for any of the repression mutants tested (Table 4). These results indicate that *FBP1* gene expression is regulated mainly by glucose derepression.

Isolation of derepression mutants. To identify more elements involved in positive regulation of the *FBP1* gene, yeast strain WAY.5-4A carrying the $UAS1_{FBP1}$-*CYC1-lacZ* fusion plasmid pDG24 was mutagenized and screened for putative mutations decreasing $UAS1_{FBP1}$-dependent expression (see Materials and Methods). From the mutants isolated, one complementation group, carrying *cat8*, showed normal respiration and was not allelic to previously described *cat1* and *cat3* derepression mutants. Five allelic *cat8* mutants were found, whereas no *cat1* or *cat3* mutants were obtained because of their inability to grow on the derepressing media used. All *cat8* mutations were recessive and showed a 2:2 segregation, which indicates a single nuclear gene mutation. The expression of the $UAS2_{FBP1}$-*CYC1-lacZ* fusion and of the *FBP1-lacZ* fusion was also affected in all *cat8* mutants isolated. Table 5 shows enzyme activities of two *cat8* strains compared with those of their isogenic parental wild-type strain, WAY.5-4A. Like *cat1* and *cat3* mutants, *cat8* mutants are unable to grow on nonfermentable carbon sources and could not derepress gluconeogenic enzymes. However, in contrast to the case for *cat1* and *cat3* mutants, regulation of the disaccharide-utilizing enzymes maltase and invertase was like that of the wild type in *cat8* mutants. This was also true for growth on sucrose and raffinose.

TABLE 5. Glucose repression and enzyme activities of the wild-type strain and *cat8* mutants

Strain[a]	Sp act[b]						
	FBPase (YEPE)	ICL (YEPE)	PEPCK (YEPE)	Invertase		Maltase	
				YEPD	YEPR	YEPD	YEPM
WAY.5-4A	48	75	216	29	741	17	1,807
WAY.5-4A/160	1	1	1	24	1,306	15	2,116
WAY.5-4A/65	2	1	4	36	1,071	19	4,603

[a] Strains are described in Table 1.
[b] Enzyme activities are expressed as nanomoles of substrate converted per minute per milligram of protein. Abbreviations: FBPase, fructose-1,6-bisphosphatase; ICL, isocitrate lyase; PEPCK, phosphoenolpyruvate carboxykinase; YEPD, yeast extract-peptone-dextrose; YEPE, yeast extract-peptone-ethanol; YEPR, yeast extract-peptone-raffinose; YEPM, yeast extract-peptone-maltose.

Cloning of *CAT8*. An *S. cerevisiae* genomic DNA library in the centromere plasmid YCp50 was transformed into the *cat8-160* mutant strain, and *URA* prototroph transformants were collected and in a second step were plated on ethanol at 30°C. Plasmid DNA was isolated from transformants growing on ethanol and then retransformed to confirm complementation. Plasmid pDG160 was found to suppress the growth defect on nonfermenting media caused by the *cat8-160* mutation. Several subclones in YCp50 or YCplac33 were assessed for complementation of the growth defect of the *cat8-160* mutant (Fig. 1). We determined the nucleotide sequence of the functional region between the *Pst*I site and the *Sna*BI site located 3′ to the gene (Fig. 1). A single open reading frame encoding 1,433 amino acids was identified (Fig. 2). The predicted Cat8p protein of 160,462 Da contains a zinc cluster motif near the N terminus which is closely related to those of the yeast positive regulatory proteins Gal4p (23) and Ppr1p (25) and the *Kluyveromyces lactis* protein Lac9p (52) (Fig. 3).

Genetic analysis of *CAT8*. To determine the phenotype caused by loss of *CAT8* gene function, we replaced most of the Cat8p-encoding region by the *HIS3* prototrophy (Fig. 1). As expected, the resulting *cat8*::*HIS3* mutant did not grow on nonfermentive carbon sources and could not derepress the gluconeogenic enzymes, whereas expression of invertase and maltase was normal (data not shown). The *CAT8* gene is located on chromosome XIII, as judged by whole-chromosome blot hybridization.

Regulatory properties of the *CAT8* gene. For *CAT8* expression analyses, plasmid pCAT8lacZ, which contained ~1,800 nucleotides of the upstream promoter sequence and the first 69 codons of the *CAT8* gene fused in frame to the β-galactosidase open reading frame, was used. As summarized in Fig. 4A, the β-galactosidase activities of wild-type cells transformed with pCAT8lacZ were strictly repressed after growth with glucose. Transfer to derepression medium led to an ~57-fold increase in β-galactosidase activity after 8 h. The derepression kinetics were similar to those of genes *FBP1* (fructose-1,6-bisphosphatase [37]), *PCK1* (phosphoenolpyruvate carboxykinase [39]) and *ICL1* (isocitrate lyase [42]). Surprisingly, these results revealed that the *CAT8* gene itself is regulated by glucose repression. To confirm this result, the increase in Cat8-LacZ fusion protein was monitored by Western blot (immunoblot) analysis during derepression (Fig. 4B). No cross-reaction occurred after growth on glucose, whereas after 1 h of derepression, a weak signal, which strongly increased after 2 h, was detected. These results confirmed that Cat8p synthesis is strongly regulated by the carbon source available.

***MIG1* (*CAT4*) is involved in glucose repression of the *CAT8* gene.** To verify the observed *CAT8* regulation, we searched for

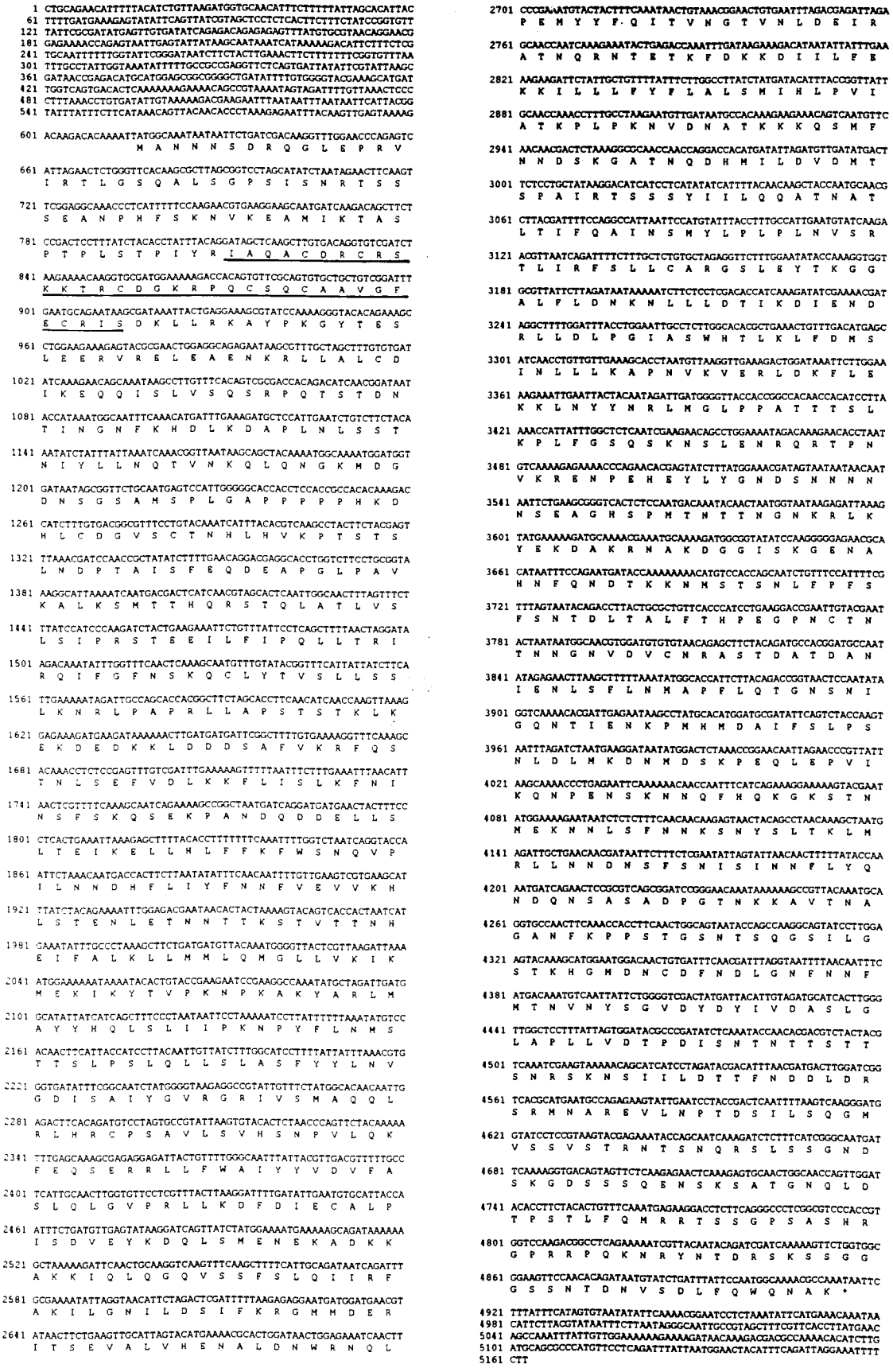

FIG. 2. Nucleotide sequence of the *CAT8* gene and deduced amino acid sequence. The zinc cluster motif is underlined.

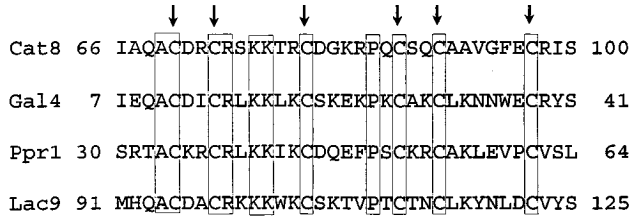

FIG. 3. Similarities of Cat8p to other zinc cluster proteins. The zinc cluster motif of Cat8p is aligned with those of the *S. cerevisiae* Gal4p and Ppr1p proteins and the *K. lactis* Lac9p protein. Boxes indicate identical residues, and arrows point to the cysteine residues that are conserved in all Cys_6-zinc cluster motifs. Numbers refer to the position of the finger motif within the amino acid sequence, starting from the amino terminus of the respective protein.

TABLE 6. Influence of regulatory factors on expression of a *CAT8-lacZ* gene

Strain[a]	Relevant genotype	Sp act of β-galactosidase (nmol/min/mg) under:	
		Repressed conditions[b]	Derepressed conditions[c]
WAY.5-4A	Wild type	29	1,648
PK18/1	*mig1* (*cat4*)	740	1,439
JS87.11-7C	*cat1*	24	63
JS87.15-7D	*cat3*	46	64

[a] The plasmid pCAT8lacZ was used for transformation.
[b] Growth in SCD_4 medium lacking uracil.
[c] Growth in SCE_3 medium lacking uracil.

possible *cis*-regulatory elements within the *CAT8* promoter. We identified a sequence element within the *CAT8* promoter region (ATTTTGTGGGG; positions −220 to −210) that perfectly matched the *MIG1* (*CAT4*) consensus motif $[(A/T)_5(G/C)(C/T)GG(A/G)G]$ (30). The well-known zinc finger protein Mig1p functions as a repressor of the *SUC* and *GAL* genes (34, 35). Previous genetic analysis has identified *cat4* (allelic to *mig1*) as a suppressor of the *cat1* (*snf1*) kinase (44). To investigate a possible role of Mig1p in *CAT8* regulation, the pCAT8lacZ reporter construct (see above) was transformed in a *cat4* mutant. As expected, a marked effect on *CAT8* glucose repression was observed (Table 6). On glucose, β-galactosidase activities were increased ~26-fold in the *cat4* mutant compared with the wild type.

The Cat1p/Cat3p protein kinase is essential for derepression of gluconeogenic genes. Therefore, we examined *CAT8* expression in *cat1* and *cat3* mutants by using the *CAT8-lacZ* construct. In both mutants the β-galactosidase activities were at a basal level in repressed and derepressed transformants (Table 6). To determine the functional relationship between *CAT8* and *CAT1*, we transformed a *cat1*::*HIS3* null mutant with multicopy plasmid pMP59 containing the *CAT8* gene. The plasmid restored growth of the *cat1*::*HIS3* null mutant on ethanol (Fig. 5), and the same result was also observed for *cat3* mutants (data not shown). Although multicopy expression of *CAT8* restored growth with ethanol for *cat1* and *cat3* mutants, no increase in the activities of gluconeogenic enzymes was observed.

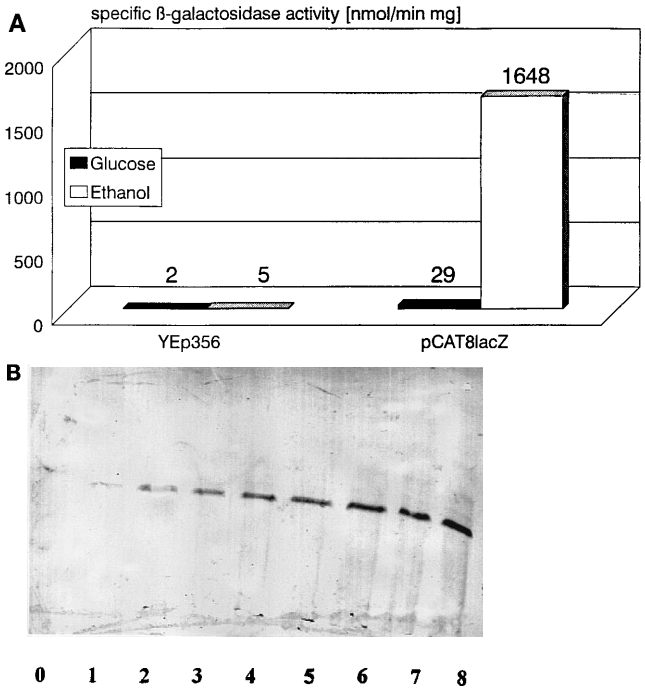

FIG. 4. Expression of the *CAT8* gene. (A) Specific β-galactosidase activities in yeast wild-type strain WAY.5-4A. All plasmids contain the 2 μm origin of replication and *URA3* as a selection marker. Plasmids are described in Materials and Methods. (B) Western blot analysis. WAY.5-4A was transformed with plasmid pCAT8lacZ. Cells grown overnight in SCD_4 medium were harvested, washed, and resuspended in SCE_3 medium. Crude extracts were prepared from cells at the times (hours after derepression) indicated at the bottom.

DISCUSSION

In *S. cerevisiae*, transcription of gluconeogenic genes is controlled by the global regulatory mechanism termed glucose repression. This work describes *cis*- and *trans*-regulatory elements necessary and sufficient for expression of the gluconeogenic fructose-1,6-bisphosphatase gene (*FBP1*) from *S. cerevisiae*. Two positively *cis*-acting motifs, $UAS1_{FBP1}$ and $UAS2_{FBP1}$, were previously characterized, and deletion of both elements resulted in a complete loss of *FBP1* derepression (37). As reported here, the insertion of each element into a heterologous, UAS-free promoter revealed a strong carbon source-dependent activation. In contrast to genes involved in disaccharide and galactose utilization (*SUC*, *MAL*, and *GAL* genes), a different mechanism of transcriptional control for the *FBP1*

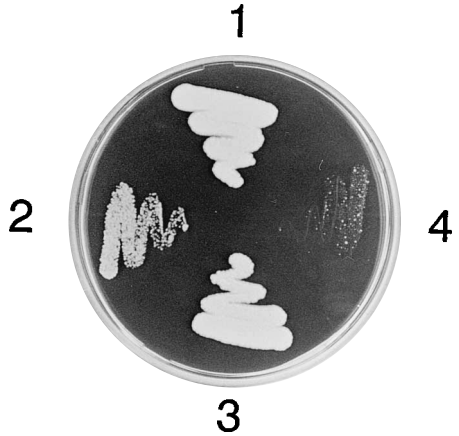

FIG. 5. Gene dosage effect of *CAT8*. Growth on yeast extract-peptone-ethanol plates at 30°C of WAY.5-4A plus pMP59 (multicopy *CAT8*) (1) *cat1*::*HIS3* plus pMP59 (multicopy *CAT8*) (2), WAY.5-4A (3), and *cat1*::*HIS3* (4) is shown.

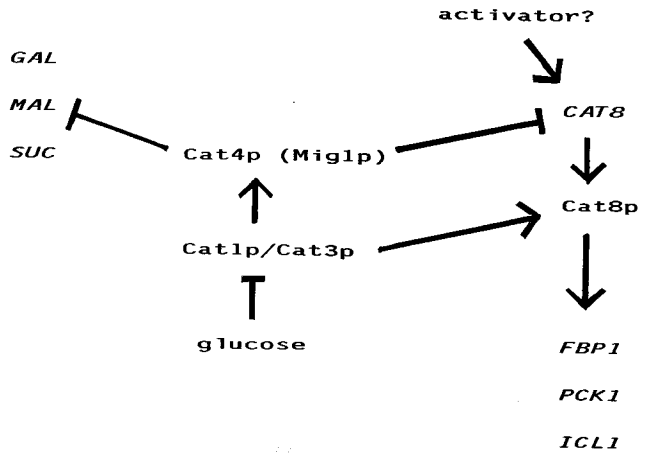

FIG. 6. Model for glucose repression and derepression.

gene and other gluconeogenic genes became obvious. Mutations *hxk2* (*hex1*), *cyc8* (*ssn6*), and *mig1* (*cat4*), which cause a defect in glucose repression of invertase, maltase, and galactose-utilizing enzymes, had no influence on either $UAS1_{FBP1}$- or $UAS2_{FBP1}$-dependent activation. This result is in agreement with previous studies in which even a strong selection system failed to isolate mutants defective in *FBP1* repression (31). Glucose repression by UAS1 and UAS2 of fructose-1,6-bisphosphatase is obviously mediated by positive *trans*-regulatory elements for gene activation.

The characterization of control regions upstream of key enzymes of gluconeogenesis and the glyoxylate cycle led to the identification of several *cis*-acting elements essential for derepression. For the *PCK1* gene, encoding the enzyme phosphoenolpyruvate carboxykinase, two UASs have been found (39). The $UAS1_{PCK1}$ and $UAS2_{PCK1}$ motifs of phosphoenolpyruvate carboxykinase behave similarly to $UAS1_{FBP1}$ and $UAS2_{FBP1}$ in heterologous test plasmids (39). For the key glyoxylate cycle enzyme isocitrate lyase, a carbon source-responsive element (positions −397 to −388; 5′-CATTCATCC G-3′) has been identified (43). Gene activation by the carbon source-responsive element requires the derepression genes *CAT1* and *CAT3*. Homologies to $UAS2_{PCK1}$ and $UAS2_{FBP1}$ within the above-mentioned sequence element have been described (43). As previously described, derepression of glucose-repressible enzymes depends on Cat1p (Snf1p) protein kinase and its subunit Cat3p (Snf4p). The failure to derepress $UAS1_{FBP1}$ and $UAS2_{FBP1}$ in *cat1* and *cat3* mutants revealed that their activation also depends on the active Cat1p/Cat3p protein kinase.

By using the $UAS1_{FBP1}$ element as a specific target for the isolation of $UAS1_{FBP1}$ derepression mutants, we could identify the *CAT8* gene, which represents the missing link in understanding of the genetics of glucose derepression. The *CAT8* gene is essential for derepression of all gluconeogenic enzymes. Sequence analysis indicated that *CAT8* encodes a Cys_6-zinc cluster protein. Related yeast proteins are the transcription activators Gal4p (23) and Ppr1p (25). *CAT8* expression studies showed a strong regulation that was dependent on the carbon source provided. Previous analysis found that the Mig1p (Cat4p) protein represses *GAL4* transcription by binding to the *GAL4* promoter (35). We found that *MIG1* (*CAT4*) also regulates *CAT8* gene expression. This result is consistent with the observation that expression of gluconeogenic enzymes is not directly influenced by *MIG1* (*CAT4*) (30, 42).

From the results obtained so far, a model for glucose repression and derepression can be derived. As shown in Fig. 6, the Cat8p zinc cluster protein is important for derepression of gluconeogenic genes. The transcription of the *CAT8* gene is subject to glucose repression with Cat4p (Mig1p) as its repressor. The Cat4p (Mig1p) zinc finger protein also represses the transcription of the *SUC* genes and the *GAL* genes. For derepression, the binding of Cat4p (Mig1p) to *CAT8*, *SUC* genes, and *GAL* genes is prevented, and consequently the respective genes are transcribed. This hypothesis is confirmed by *cat4* (*mig1*) mutants which do not repress invertase, *GAL* genes, and *CAT8*. However, the Cat8p synthesis is not sufficient for derepression of gluconeogenic enzymes, as their synthesis is still glucose repressible in *cat4* (*mig1*) mutants. So far we cannot absolutely exclude the possibility that other repressors bind to *FBP1* which could be inhibited by Cat1p and therefore repress gluconeogenic gene expression in a *cat4* (*mig1*) mutant background. *CAT8* expression also depends on the Cat1p/Cat3p protein kinase. This is the same for *SUC* and *GAL* genes. These genetic results indicate that Cat4p is negatively modified by the Cat1p/Cat3p protein kinase. From the present data we conclude that under conditions of glucose repression, Cat4p (Mig1p) binds as a repressor to *SUC* genes, *GAL* genes, and *CAT8*, thus preventing their transcription. After glucose consumption, the Cat1p/Cat3p protein kinase is activated and converts Cat4p (Mig1p) to a nonbinding conformation, possibly by its phosphorylation. Consequently, *SUC* genes, *GAL* genes, and *CAT8* are transcribed. For derepression of gluconeogenic genes, the Cat1p/Cat3p protein kinase converts the Cat8p protein to a binding conformation, possibly by its phosphorylation. This hypothesis is supported by findings that *CAT8* in multicopy suppressed the growth defect of *cat1* and *cat3* mutants on ethanol. The activation of key gluconeogenic enzymes by binding of Cat8p directly to the UAS sequences is most likely, but so far we cannot exclude the possibility that Cat8p acts indirectly or together with so-far-unknown proteins.

The model presented here is conclusive for the interpretation of the genetic results obtained so far. However, we assume that other proteins in addition to Cat8p are involved in derepression of gluconeogenic genes. This became obvious with *cat1 cat4* double mutants which did not derepress *CAT8*, suggesting that in addition to the prevention of binding to Mig1p (Cat4p), further activators are necessary for *CAT8* transcription. This is an important difference from *SUC* genes, which are derepressed in a *cat1 cat4* double mutant.

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