MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2502–2510 Vol. 17, No. 5
0270-7306/97/$04.00+0

Glucose Derepression of Gluconeogenic Enzymes in *Saccharomyces cerevisiae* Correlates with Phosphorylation of the Gene Activator Cat8p

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Received 21 October 1996/Returned for modification 3 December 1996/Accepted 11 February 1997

The Cat8p zinc cluster protein is essential for growth of *Saccharomyces cerevisiae* with nonfermentable carbon sources. Expression of the *CAT8* gene is subject to glucose repression mainly caused by Mig1p. Unexpectedly, the deletion of the Mig1p-binding motif within the *CAT8* promoter did not increase *CAT8* transcription; moreover, it resulted in a loss of *CAT8* promoter activation. Insertion experiments with a promoter test plasmid confirmed that this regulatory 20-bp element influences glucose repression and derepression as well. This finding suggests an upstream activating function of this promoter region, which is Mig1p independent, as Δ*mig1* mutants are still able to derepress the *CAT8* promoter. No other putative binding sites such as a Hap2/3/4/5p site and an Abf1p consensus site were functional with respect to glucose-regulated *CAT8* expression. Fusions of Cat8p with the Gal4p DNA-binding domain mediated transcriptional activation. This activation capacity was still carbon source regulated and depended on the Cat1p (Snf1p) protein kinase, which indicated that Cat8p needs posttranslational modification to reveal its gene-activating function. Indeed, Western blot analysis on sodium dodecyl sulfate-gels revealed a single band (Cat8pI) with crude extracts from glucose-grown cells, whereas three bands (Cat8pI, -II, and -III) were identified in derepressed cells. Derepression-specific Cat8pII and -III resulted from differential phosphorylation, as shown by phosphatase treatment. Only the most extensively phosphorylated modification (Cat8pIII) depended on the Cat1p (Snf1p) kinase, indicating that another protein kinase is responsible for modification form Cat8pII. The occurrence of Cat8pIII was strongly correlated with the derepression of gluconeogenic enzymes (phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase) and gluconeogenic *PCK1* mRNA. Furthermore, glucose triggered the dephosphorylation of Cat8pIII, but this did not depend on the Glc7p (Cid1p) phosphatase previously described as being involved in invertase repression. These results confirm our current model that glucose derepression of gluconeogenic genes needs Cat8p phosphorylation and additionally show that a still unknown transcriptional activator is also involved.

Nonfermentative growth of *Saccharomyces cerevisiae* by using a C_2 or C_3 energy substrate (ethanol, acetate, lactate, or glycerol) requires gluconeogenic enzyme activities. Enzymes belonging to the gluconeogenic pathway are strictly regulated depending on the available carbon source. Transcriptional regulation of gluconeogenic genes by glucose repression is the most important mechanism for long-term adaptation of the enzyme levels in response to fermentation or respiration conditions.

The complex glucose repression regulatory system also affects the transcription of genes involved in the utilization of alternative sugars (*SUC*, *MAL*, or *GAL* genes), the Krebs cycle, oxidative phosphorylation, peroxisomal biogenesis, and β-oxidation (for reviews, see references 10, 16, 21, and 41). Concerning the gluconeogenic and glyoxylate cycle pathways, target genes of glucose repression are *FBP1* (encoding fructose-1,6-bisphosphatase [FBPase]), *PCK1* (encoding phosphoenolpyruvate carboxykinase [PEPCK]), *ICL1* (encoding isocitrate lyase), and *MLS1* (encoding gluconeogenic malate synthase). Several studies on the 5′ regulatory sequences of these genes indicated a coregulatory mechanism acting on common *cis* elements within the promoters of gluconeogenic and glyoxylate cycle genes. Upstream activation sites (UAS) were identified in the *FBP1* and *PCK1* promoters (20, 37–39) as the essential *cis* activators for the release from glucose repression. The consensus UAS motif CGGRYRNMNGGA (CSRE [carbon source-responsive element] [44]) was also found in the *ICL1* gene. Furthermore, carbon source-dependent protein binding to the UAS/CSRE (37, 44) suggested these sequences to be the final targets of a glucose-regulated gene activation.

Activation (derepression) of gluconeogenic genes through UAS/CSRE requires the function of *CAT1* (*SNF1*), *CAT3* (*SNF4*), and *CAT8* genes (20, 39, 44). Cat1p is a serine/threonine protein kinase (3), with Cat3p (45) as a physically associated subunit necessary for full kinase activity (4, 12). The pleiotropic Cat1p-Cat3p kinase complex is involved in the activation of a wide variety of glucose-repressible genes, including those of the utilization of galactose, di- and trisaccharides (*GAL*, *SUC*, and *MAL* genes), gluconeogenesis, and respiration (9, 57). Several proteins (Sip1p, Sip2p, Gal83p, Sip3p, and Sip4p) have been identified by their interaction with the Cat1 protein. Therefore, these factors have been proposed either to direct the Cat1p kinase function to specific pathways or to be the Cat1p substrates directly involved in gene activation. The Sip1/Sip2/Gal83 protein family plays a role in mediating repression by glucose to the *GAL* genes (11, 34, 54, 55). Sip3p and Sip4p both interact with Cat1p and show transcriptional

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

Strain	Genotype
WAY.5-4A	*MAT***a** *ura3-52 his3-Δ1 MAL2-8^c MAL3 SUC3*
CEN.PK2-1C	*MAT***a** *ura3-52 his3-Δ1 leu2-3,112 trp1-289 SUC2*
CEN.NB1-1A	*MAT***a** *ura3-52 his3-Δ1 leu2-3,112 trp1-289 cat8::LEU2 SUC2*
CEN.PK130-7B	*MATα ura3-52 his3-Δ1 leu2-3,112 trp1-289 cat1::HIS3 SUC2*
CEN.Δmig1-1A	*MAT***a** *ura3-52 his3-Δ1 leu2-3,112 trp1-289 mig1::loxp-KAN-loxp SUC2*
CEN.Δmig1Δcat1-1B	*MATα ura3-52 his3-Δ1 leu2-3,112 trp1-289 mig1::loxp-KAN-loxp cat1::HIS3 SUC2*
ENY.cid1-9C	*MATα ura3-52 leu2-3,112 ade2 cid1-226 MAL2-8^c MAL3 SUC3*
SFY526	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can^r gal4-542 gal80-538 URA3::GAL1-lacZ*

activation capacities (27, 28), but in none of these cases has the participation in regulation of a specific gene been demonstrated. The previously isolated *CAT8* gene is specifically required for UAS/CSRE-mediated activation of gluconeogenic genes (20). Cat8p belongs to the family of transcriptional activators containing a binuclear C_6 zinc cluster. *CAT8* expression itself is regulated by glucose repression. The general zinc finger regulator Mig1p (Cat4p) (35) is responsible for transcriptional repression of *CAT8*, whereas the Cat1p-Cat3p kinase function is essential for its transcriptional activation (20). From genetic data, it was presumed that the Cat1p-Cat3p kinase complex does not simply influence *CAT8* expression but in addition activates Cat8p posttranslationally (20, 39).

Since the generation of an active Cat8p transcriptional factor seemed to be the central step in the derepression process of gluconeogenesis, we investigated both the transcriptional regulation of the *CAT8* gene and the activation of the Cat8 protein.

MATERIALS AND METHODS

Strains and growth conditions. The *S. cerevisiae* strains used are listed in Table 1. For amplification of recombinant plasmids, *Escherichia coli* DH5α [(ϕ*80d*/*lacZ*ΔM15) Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17 supE44 thi-gyrA96 relA1*] was used.

The composition of synthetic complete (SC) medium has been previously described (36). As a carbon source, 4% glucose, 2% maltose, 2% raffinose, 2% galactose, or 3% ethanol was added. For derepression, cells were grown in glucose medium to mid-log phase and then transferred to medium with 3% ethanol as the carbon source for 4 to 6 h.

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

Plasmid constructs. To characterize *cis*-acting regulatory sequences, plasmid pJS205 was used (46). The 3′-shortened PCR fragments were inserted in *Hin*dIII/*Xho*I-treated pJS205. To obtain the pCAT8 plasmids, the resulting constructs were digested with *Hin*dIII and *Pst*I, mung bean nuclease treated, and self-ligated. The correct integration of the inserts was determined by DNA sequencing. In pNB37, the PCR fragment was inserted into the single *Xho*I site of pJS205. To obtain pNB40, 5′-phosphorylated and hybridized synthetic oligonucleotides (ARK Scientific) ONB27 and ONB28 were used. They were synthesized with a control restriction site (*Bgl*II) and to result in *Xho*I-compatible ends which were used for insertion into *Xho*I-cleaved pJS205. The orientations and number of inserts were determined by DNA sequencing. The construction pNB2 was derived from the insertion of the 817-bp *Pst*I/*Hin*dIII fragment of the *CAT8* promoter into YEp356 (31). Plasmid pNB2 contains 616 bp of the *CAT8* promoter and the first 67 codons of *CAT8*, fused in frame to β-galactosidase. The *CAT8* promoter sequence 5′-ATATTTTGTGGGG-3′ (positions −210 to −222) was exchanged for 5′-AGATCT-3′ by PCR. The resulting construct was verified by DNA sequencing. To characterize transcriptional activating properties of Cat8p, plasmid pGBT9 (Clontech) was used. PCR fragments carrying different parts of *CAT8* were inserted into *Sal*I/*Pst*I-digested pGBT9. YCplac111*GALlacZ* (kindly provided by H. Juhnke, Frankfurt am Main) was used as a reporter construct for estimation of transcriptional activation. It is derived from YCplac111 (17) and contains the *GAL1* promoter fused to *lacZ*. Plasmids pMP59 and pMP121 are described in references 20 and 39, respectively.

Yeast transformation. All plasmids used in this study were transformed into yeast as described elsewhere (23). Transformants were selected on SC medium lacking the appropriate amino acids or uracil (1).

Enzyme assays. Crude extracts were prepared with glass beads (6), and protein was determined by the microbiuret method (56) at 290 nm with bovine serum albumin as a standard. β-Galactosidase activity was measured as described previously (18). Specific activities are the averages of four independent transformants measured in duplicate. In all cases, the standard error was less than 10%.

λ-Phosphatase treatment. Protein samples were extracted in buffer (50 mM Tris-HCl [pH 7.5], 2 mM $MnCl_2$) containing a mixture of protease inhibitors (pepstatin, leupeptin, and aprotinin [each at 1 μg/ml] and 1 mM phenylmethylsulfonyl fluoride). Crude extracts (50 μg per reaction) were incubated with 200 U of λ-phosphatase (New England Biolabs) in a volume of 20 μl for 1 h at 30°C. Samples containing 0.1 mM Na_3VO_4, a specific λ-phosphatase inhibitor, were treated identically. Control reactions were incubated on ice. Reactions were terminated by addition of 10 μl of 2× Laemmli sample buffer and heating to 100°C for 3 min.

Northern analysis. Cells growing exponentially in SCD_4 medium were transferred to SCE_3 medium. At different times, cells were harvested and total RNA was extracted as described previously (48). Northern analysis of the samples was carried out as described previously (43), using fragments of *PCK1* and *ACT1* genes as probes. Probes were obtained by digesting plasmids containing the corresponding genes with appropriate restriction enzymes (a 1.2-kb *Xho*I/*Hin*dIII fragment from plasmid pACTIN [13] and a 1.6-kb *Eco*RI/*Pst*I fragment from plasmid pPEPCK [kindly provided by M. Rose]). A Ready-to-Go DNA labelling kit (Pharmacia Biotech Europe GmbH) was used for [^{32}P]dCTP radiolabelling for the DNAs.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 6 or 12% polyacrylamide gels, using the buffer system described in reference 25. Western transfer of proteins to a nitrocellulose filter was carried out as described previously (52). Cat8p, Pck1p, and Fbp1p were detected by incubation with polyclonal antisera (dilution of 1:1,000) and alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma). 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 $MgCl_2$). Polyclonal antiserum against Cat8p was obtained by the standard immunization protocol (Eurogentec), using a purified TrpE-Cat8 fusion protein which comprised amino acids 134 to 460 from Cat8p.

Antisera against Pck1p and Fbp1p were generated by using the purified proteins. $Gal4_{BD}$ (Gal4p DNA-binding domain) Cat8 fusion protein (expressed from pMP151) was detectable only after enrichment of the hybrid protein by immunoprecipitation with Cat8p antibody and *Staphylococcus aureus* cells (Pansorbin; Calbiochem). Immunodetection was carried out by using monoclonal anti-$Gal4_{BD}$ antiserum (dilution of 1:3,200; Clontech) and peroxidase-conjugated anti-mouse antibody (dilution of 1:2,000; Amersham). Bound antibody was detected by using the ECL chemiluminescence system (Amersham) as instructed by the manufacturer.

RESULTS

Characterization of the *CAT8* promoter. By genetic studies, we previously showed that *MIG1* (*CAT4*) is involved in glucose repression of *CAT8* and that the Cat1p-Cat3p protein kinase is essential for derepression of the *CAT8* gene (20). In fact, a motif for the negative transregulator Mig1p (ATATTTTGTG GGG) is located between positions −210 and −222 in the upstream region of *CAT8*, which perfectly fulfills the criteria found for Mig1p binding (28a). In addition to this element, two putative binding sites for transcriptional activators were characterized by sequence comparison in the *CAT8* promoter: a possible binding site (TGATTGGT) for the heteromeric Hap2/3/4/5p complex was suggested to exist between positions −192 to −199 (40); between positions −252 and −264, a sequence element (CGTATTAAGCGAT) which perfectly matched the consensus sequence of the autonomously replicating sequence-binding factor and general transcriptional activator Abf1p ($RTCRYN_5ACG$ [8]) was found.

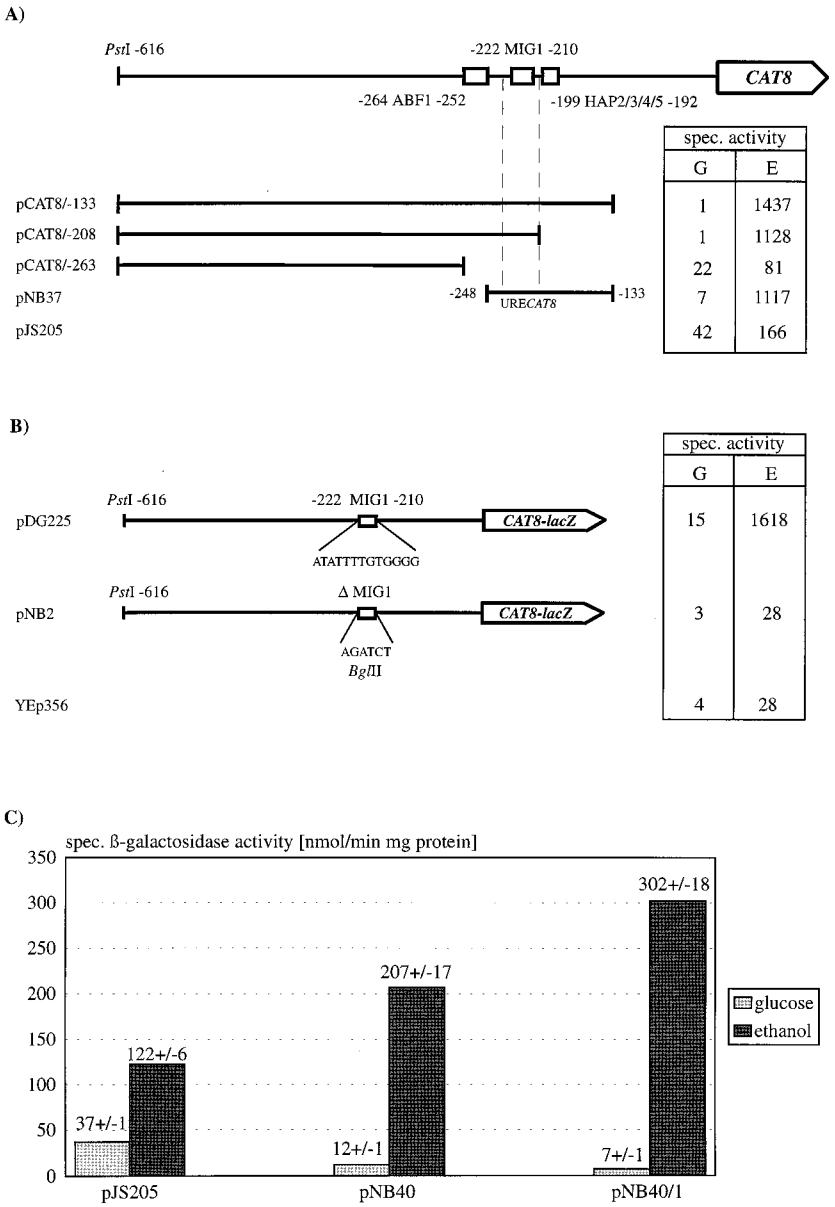

FIG. 1. Deletion analysis of the *CAT8* promoter. Transformed wild-type cells (WAY.5-4A) were assayed under repressed (SCD_4 medium lacking uracil [G]) and derepressed (SCE_3 medium lacking uracil [E]) growth conditions. β-Galactosidase (β-gal.) specific activities are given in nanomoles per minute per milligram of protein. (A) Insertion of *CAT8* promoter fragments into the UAS-less test plasmid pJS205. (B) Expression of *CAT8-lacZ* fusions. Construct pDG225 contains the complete *CAT8* upstream region up to position −616. The corresponding construct pNB2 lacks the Mig1p-binding site. (C) Insertion of a synthetic oligonucleotide representing URE_{CAT8} into pJS205. pNB40 and pNB40/1 contain a single and double insertions, respectively.

To identify positively acting *cis*-regulatory elements in the upstream region of *CAT8*, we inserted shortened *CAT8* promoter fragments with 3′ deletions in the promoter test plasmid pJS205 (46) (Fig. 1A). This episomal plasmid lacks UAS elements and allows only basal expression of a *CYC1-lacZ* reporter gene. After insertion of the promoter fragments, a carbon source-dependent induction of the *lacZ* expression could be observed with constructs pCAT8/−133 and pCAT8/−208. On glucose, the insertion of the promoter fragments −133 to −616 (pCAT8/−133) and −208 to −616 (pCAT8/−208) led to a 42-fold decrease of β-galactosidase activity in comparison to pJS205, which can be due to the presence of the Mig1p-binding site in both fragments. After derepression, β-galactosidase activity increased more than 1,000-fold with both constructs, whereas test plasmid pJS205 exhibited only a 4-fold increase in β-galactosidase activity. These results show that the −208 to −616 region is sufficient for derepression of the *CAT8* promoter. According to these results, a direct positive transregulation through Hap2/3/4/5p became unlikely, because its putative binding site is not present in the pCAT8/−208 construct. After insertion of the promoter fragment −263 to −616 (pCAT8/−263), the resulting construct was not able to derepress β-galactosidase activity, suggesting the existence of a UAS element between positions −208 and −263. This region comprises the putative Abf1p-binding motif. However, the insertion of the promoter fragment −133 to −248, lacking the

Abf1p-binding site (pNB37), revealed the same carbon source-dependent regulation as pCAT8/−133 and pCAT8/−208 constructs, which indicates that Abf1p is also not involved in *CAT8* expression (Fig. 1A). Comparison of plasmids pCAT8/−208 and pNB37 reveals that the region responsible for carbon source-dependent regulation is localized between positions −248 and −208. This region includes the previously described Mig1p-binding site (20).

As shown by the non-glucose-repressible *CAT8* expression in *mig1* mutants (20), Mig1p clearly acts as a repressor of *CAT8* transcription. To determine if the integrity of this binding site is important for *CAT8* expression, we deleted the Mig1p-binding site in a *CAT8-lacZ* fusion (pNB2). Surprisingly, this deletion resulted in a complete loss of promoter function (Fig. 1B). These data suggest that the Mig1p-binding site in the *CAT8* promoter is identical to, or at least overlaps, a target site for *CAT8* gene activation.

To further characterize the regulatory function of the putative Mig1p-binding site, we tested the regulatory properties of a 20-bp fragment (URE_{CAT8}) containing the Mig1p-binding site. This synthetic URE_{CAT8} fragment, which represents positions −204 to −223 (GATATTTTGTGGGGTACGAA) of the native *CAT8* promoter, was inserted in promoter test plasmid pJS205, resulting in constructs carrying a single (pNB40) or a double (pNB40/1) insertion. As expected, insertion of URE_{CAT8} led again to a strict carbon source-dependent expression of β-galactosidase (Fig. 1C). In correlation with the *CAT8* promoter deletion results, URE_{CAT8} behaved negatively under repressing conditions and positively under derepressing conditions. More than that, the double insertion of the oligonucleotide (pNB40/1) further strengthened the reported effects, thus supporting the hypothesis that repression and activation of the *CAT8* promoter act via adjacent target sequences or possibly the same target sequence.

Transcriptional activation by Cat8p is dependent on Cat1p function. Different *CAT8* fragments were fused to $GAL4_{BD}$ to test their abilities to activate a Gal4p-dependent *GAL1-lacZ* reporter gene. All $GAL4_{BD}$-*CAT8* fusion genes were constitutively expressed from an *ADH1* promoter. As shown in Fig. 2A, C-terminal *CAT8* fragments were not able to stimulate expression of the test construct. However, a $Gal4_{BD}$-Cat8 hybrid protein containing nearly the entire Cat8p (pMP151) markedly activated transcription. Moreover, this activation property was strictly dependent on gluconeogenic growth, since it could not be detected in glucose-grown cells. These results indicate that Cat8p mediates either directly or indirectly transcriptional activation and is dependent on the carbon source provided. Furthermore, these results suggest a posttranslational modification of Cat8p, as the fusion is constitutively expressed. To test whether the pleiotropic Cat1p protein kinase is required for Cat8p activation, we assayed *GAL1-lacZ* expression by the $Gal4_{BD}$-Cat8 hybrid activator in a *cat1* deletion mutant. As shown in Fig. 2B, the carbon source-dependent activation was nearly abolished in a *cat1* deletion strain. This result suggested that possibly the Cat1p protein kinase function acts indirectly or directly on the Cat8p activator. By immunodetection of $Gal4_{BD}$-Cat8, we ruled out the possibility that the differences observed were the result of a different expression or stability of the fusion protein. As shown in Fig. 2C, the $Gal4_{BD}$-Cat8 fusion protein (expressed from pMP151) was stable under repressed and derepressed growth conditions in wild-type as well as Δ*cat1* cells. Moreover, the $Gal4_{BD}$-Cat8 protein appeared in several forms in wild-type cells, in contrast to *cat1* mutants after derepression, suggesting the fusion protein as a possible target for posttranslational modification (see below).

A

Plasmid	*CAT8* fused to $GAL4_{BD}$	Spec. ß-galactosidase activity: Glucose	Ethanol
pGBT9	GAL4BD	6	19
pMP120	GAL4BD — 649 1433	7	18
pMP126	GAL4BD — 399 1433	5	19
pMP127	GAL4BD 10 462	2	15
pNB27	GAL4BD 10 946	2	36
pMP151	GAL4BD 10 1433	9	341

B

Relevant genotype	$GAL4_{BD}$-*CAT8*	Spec. ß-galactosidase activity: Glucose	Ethanol
Wild-type	-	4	13
Wild-type	+	45	322
Δ*cat1*	-	4	7
Δ*cat1*	+	49	79

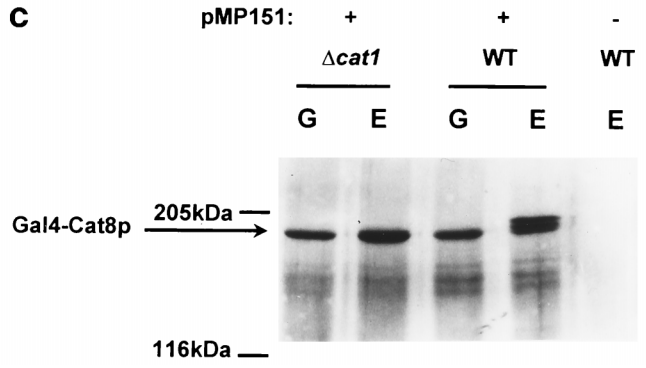

FIG. 2. Transcriptional activation of $Gal4_{BD}$-Cat8 fusion proteins. Specific β-galactosidase activities (nanomoles per minute per milligram of protein) were measured in transformants grown in selective medium containing 4% glucose or 3% ethanol. (A) Different *CAT8* fragments are indicated with respect to the amino acids fused to $Gal4_{BD}$. Fusion plasmids were transformed into SFY526. (B) CEN.PK2-1C (wild type) and CEN.PK130-7A (*cat1*) were transformed with a centromeric *GAL1-lacZ* fusion gene. To measure $GAL4_{BD}$-*CAT8* expression, plasmid pMP151 was used. (C) Immunoblot analysis of $Gal4_{BD}$-Cat8 fusion protein expressed from pMP151. Wild-type (WT) strain CEN.PK2-1C and *cat1* deletion strain CEN.PK130-7A were used. The hybrid protein was detected by using a monoclonal anti-$Gal4_{BD}$ antibody after separation of proteins by SDS-PAGE (6% polyacrylamide). The untransformed wild-type strain served as a control. Positions of molecular weight markers are given at the left.

Cat8p is posttranslationally modified upon derepression. Immunoblot analysis of crude extracts from derepressed wild-type cells (CEN.PK2-1C) overexpressing *CAT8* revealed multiple bands of Cat8p (Cat8pI, Cat8pII, and Cat8pIII) upon SDS-PAGE (Fig. 3A). This result suggests that the protein undergoes posttranslational modifications. Two of these bands (Cat8pI and Cat8pII) were detectable in glucose-grown cultures, and an additional band (Cat8pIII) with lower electrophoretic mobility appeared after ethanol incubation.

To further investigate the carbon source-dependent modification of Cat8p, we analyzed its presence in different glucose repression mutants (Fig. 3A). In a *mig1* deletion mutant, the amount of Cat8p after growth on glucose was much higher

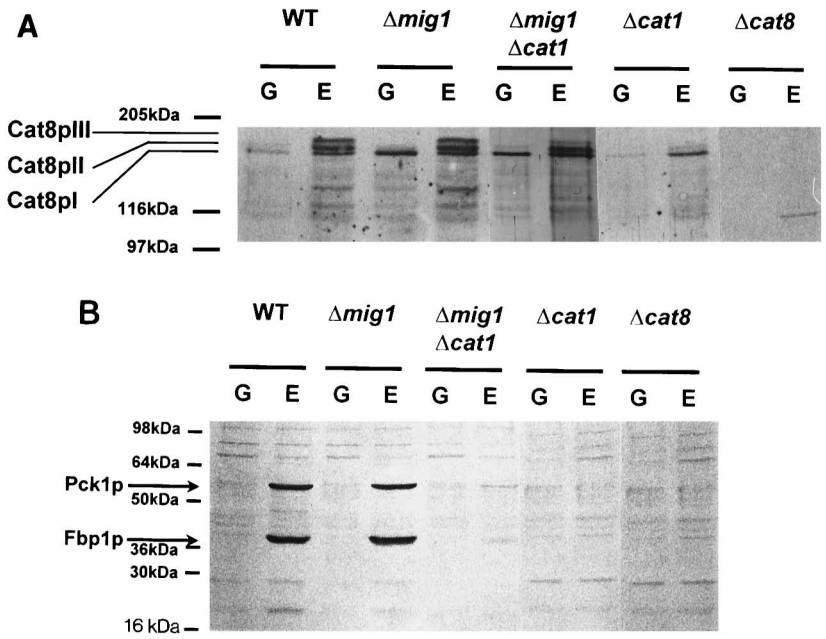

FIG. 3. (A) Immunoblot analysis reveals carbon source- and Cat1p-dependent alterations in Cat8p leading to Cat8pI, Cat8pII, and Cat8pIII with different electrophoretic mobilities. Except for the Δ*cat8* strain, cells were transformed with multicopy *CAT8* (pMP59). Cells were grown selectively under repressed conditions (G [SCD_4]) and were derepressed by a shift to ethanol-containing medium (E [SCE_3]). Proteins (50 μg per lane) were separated by SDS-PAGE in 6% polyacrylamide and subjected to immunoblot analysis using polyclonal anti-TrpE-Cat8p antiserum. Positions of molecular weight markers are indicated at the left. The strains used were CEN.PK2-1C (wild type [WT]), CEN.Δmig1-1A (Δ*mig1*), CEN.Δcat1Δmig1-1B (Δ*cat1* Δ*mig1*), CEN.PK130-7B (Δ*cat1*), and CEN.NB1-1A (Δ*cat8*). (B) Immunodetection of Pck1p and Fbp1p. Strains and growth conditions were the same as for panel A. SDS-PAGE was performed in 12% polyacrylamide gels. Polyclonal antisera against Pck1p and Fbp1p were used to detect gluconeogenic enzyme levels.

than that in wild-type cells. However, the pattern of Cat8p bands under any condition was the same as for wild-type cells. This result indicates that Mig1p is involved in modulating the abundance of Cat8p but not in its posttranslational modification. In a *cat1* deletion strain, the Cat8pIII form was not detectable after derepression, whereas the Cat8pII form appeared as a very weak band. This observation was confirmed by analysis of a *cat1 mig1* double mutant which has an increased *CAT8* expression. In this case, a very faint band corresponding to Cat8pIII was detected. Possibly, because of the stronger *CAT8* expression in a *cat1 mig1* double mutant, the Cat8pIII modification results from cross talk with other protein kinases. Interestingly, the modification forms were also observed for a $Gal4_{BD}$-Cat8 fusion protein which was immunologically tested with $Gal4_{BD}$ antibodies (Fig. 2C). This result also confirms the posttranslational modification of Cat8p.

Cat8p is a phosphorylated protein. It has been shown that phosphorylated proteins may appear as closely migrating bands upon SDS-PAGE (51). To determine whether the electrophoretic heterogeneity of Cat8p was attributable to phosphorylation, we treated crude extracts with λ-phosphatase in the presence or absence of vanadate, a specific inhibitor of the phosphatase function. Phosphatase treatment of crude extracts from derepressed cells resulted in a single band corresponding to Cat8pI (Fig. 4). Moreover, the disappearance of Cat8pII and Cat8pIII bands correlated with an increase of the Cat8pI band, which indicates that dephosphorylation converted Cat8pII and -III to Cat8pI. These data were consistent with the hypothesis that Cat8p exhibits different phosphorylation states in ethanol medium, with Cat8pIII representing a more extensively phosphorylated modification than Cat8pII. Cat8pI was not affected by phosphatase treatment (Fig. 4), and therefore we assume that this is the unphosphorylated form of Cat8p.

Cat8p phosphorylation pattern correlates with the levels of gluconeogenic enzymes. Since Cat8p is specifically phosphorylated in ethanol medium, we assayed the coincidence of gluconeogenic enzyme expression and Cat8p modification. For this purpose, we determined the levels of PEPCK and FBPase together with the Cat8p phosphorylation pattern. Immunoblot analysis (Fig. 3B) showed that in the wild type and a *mig1* mutant, PEPCK and FBPase were present to the same extent, whereas only low amounts were detectable in a *cat1 mig1* strain. The same results were obtained when *CAT8* was constitutively overexpressed (data not shown), indicating that the posttranslational modification of Cat8p is essential for glu-

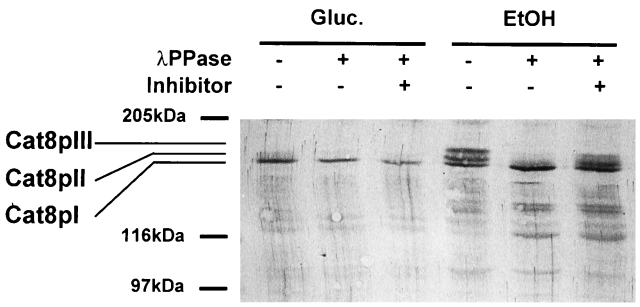

FIG. 4. Cat8pII and Cat8pIII are phosphorylated modifications of Cat8p. Protein extracts from wild-type cells (CEN.PK2-1C) expressing *CAT8* from a multicopy plasmid (pMP59) were treated with λ-phosphatase. SDS-PAGE and immunoblot analysis were as indicated for Fig. 3A. Repressed cells were grown with 4% glucose; derepression conditions were achieved by a shift to medium containing 3% ethanol (EtOH). Phosphatase reactions were done on 50 μg of total protein at 37°C for 1 h. The addition of λ-phosphatase and inhibitors is indicated above each lane.

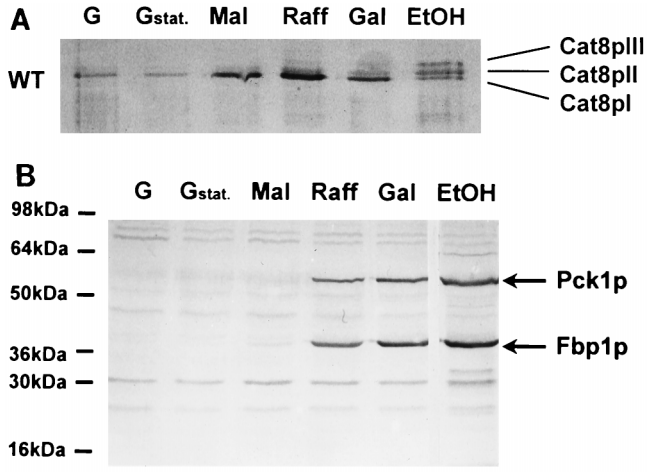

FIG. 5. (A) Phosphorylation of Cat8p is dependent on the carbon source provided to the cells. Wild-type (WT) cells (CEN.PK2-1C) expressing *CAT8* from pMP59 were grown on selective synthetic medium containing 4% glucose (G), maltose (Mal), raffinose (Raff), or galactose (Gal) or 3% ethanol (EtOH). G_{stat} indicates cells grown to stationary phase. Immunoblotting analysis was performed as indicated for Fig. 3A. (B) Levels of gluconeogenic enzymes PEPCK and FBPase are correlated with Cat8p phosphorylation. Pck1p and Fbp1p were detected by Western blot analysis in wild-type cells under the conditions used for panel A. Immunoblotting analysis was performed as indicated for Fig. 3B.

coneogenic gene activation. The presence of the most extensively phosphorylated Cat8pIII form directly correlated with the level of the gluconeogenic enzymes PEPCK and FBPase. This fact probably explains the absence of transcriptional activation found in the monohybrid experiment in a *cat1* mutant, where Cat8pIII is not present (Fig. 2C).

To show that the phosphorylation of Cat8p is a carbon source-regulated process, we examined the effects of different carbon sources on the ratio of phosphorylated forms. Cells growing on glucose medium exhibited mainly Cat8pI but also Cat8pII, and even in early-stationary-growth phase, no Cat8pIII form was detectable (Fig. 5A). The abundance of these bands was increased in cells grown on maltose, but no further phosphorylation (Cat8pIII) was detected. In contrast, on raffinose or galactose medium, Cat8pII and Cat8pIII were detectable to a minor extent (Fig. 5A). In ethanol, the phosphorylated Cat8p forms were markedly enriched. In all cases the PEPCK and FBPase levels detected by immunoblot analysis (Fig. 5B) directly correlated with the presence of the Cat8pIII form. In cells grown with raffinose, galactose, or ethanol, elevated gluconeogenic enzyme levels were found, with maximal expression in ethanol medium. These results also confirmed that Cat8pIII is indispensable for gluconeogenic gene expression.

Cat8p phosphorylation is essential but not sufficient for *PCK1* derepression. We investigated the kinetics of Cat8p phosphorylation in cells transferred from glucose- to ethanol-containing media. Cat8pIII was observed after 30 to 60 min of derepression (Fig. 6A). In parallel, we monitored the mRNA induction of *PCK1* (encoding PEPCK) by Northern blotting (Fig. 6B). The *PCK1* transcript was detectable after 2 h of ethanol incubation. These data show that Cat8p phosphorylation precedes *PCK1* expression, which is in agreement with our hypothesis that Cat8p needs phosphorylation to function as a transcriptional activator. To investigate whether the Cat8p modifications and/or *PCK1* mRNA induction are dependent on protein synthesis, cells were incubated on ethanol medium concurrently with cycloheximide (100 μg/ml). The Cat8p phosphorylation pattern was the same as in cells which were not treated with cycloheximide (Fig. 6A). In the absence of protein synthesis, Cat8pII and -III seemed to be formed at the expense of Cat8pI. Similar results were described previously for Gal4p (32), another transcriptional activator which needs to be activated by phosphorylation. However, *PCK1* mRNA was not detectable in Northern blot analyses using cells treated with cycloheximide, even when Cat8p was overexpressed with its own promoter (pMP59) or with the constitutive actin promoter (pMP121) (data not shown). These results suggest the pres-

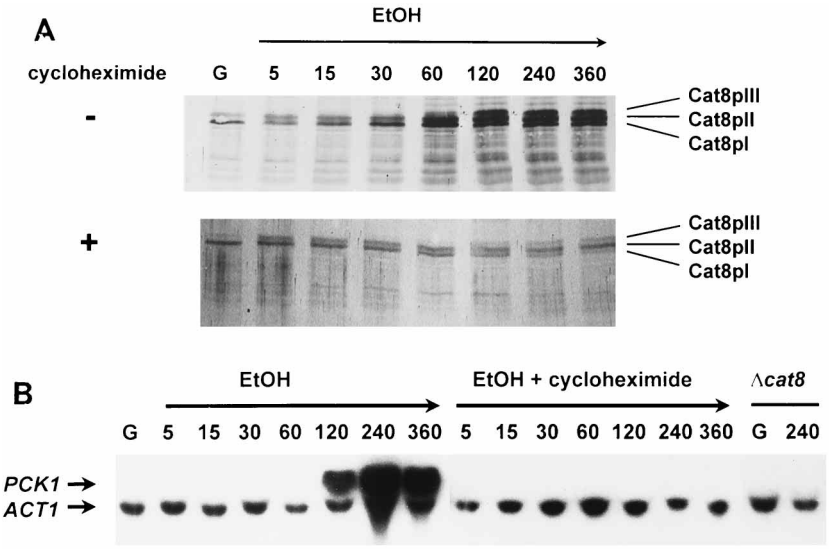

FIG. 6. Kinetics of Cat8p phosphorylation and of *PCK1* mRNA induction. Wild-type cells (CEN.PK2-1C) expressing *CAT8* from a multicopy plasmid (pMP59) were grown in glucose (G) and then transferred to ethanol (EtOH)-containing medium without and with 100 μg of cycloheximide per ml. The time scale is indicated in minutes. (A) Cat8p phosphorylation detected by Western blotting as indicated for Fig. 3A. (B) *PCK1* mRNA induction analyzed by Northern blotting. In the last two lanes, the untransformed *cat8* deletion strain CEN.NB1-1A was used.

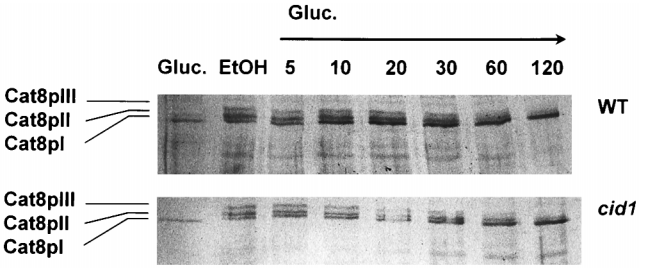

FIG. 7. Cat8p phosphorylation is sensitive to glucose. The wild type (WT; CEN.PK2-1C) and a *cid1* (*glc7*) mutant (ENY.cid1-9C) harboring multicopy *CAT8* (pMP59) were grown with 4% glucose to the mid-log phase (Gluc.) and derepressed for 8 h with 3% ethanol (EtOH). After the cells were transferred again to glucose-containing medium, samples were taken at the indicated times. Proteins (50 μg per lane) were separated by SDS-PAGE and analyzed by immunoblotting as indicated for Fig. 3A.

ence of other proteins that are the direct activators of gluconeogenic genes.

Glucose triggers dephosphorylation of Cat8p. The addition of glucose to cells growing on a nonfermentable carbon source triggers a variety of regulatory phenomena, including inactivation of gluconeogenic enzymes by reversible phosphorylation (26, 30), proteolytic degradation of the proteins (14, 15, 19), and transcriptional repression of the gluconeogenic genes (19, 47). To investigate the response of Cat8p modifications to glucose, we transferred cells grown in ethanol medium to a medium containing 4% glucose. As shown in Fig. 7, glucose induced the disappearance of Cat8pIII, with a simultaneous increase of Cat8pI. The level of *CAT8* mRNA declined rapidly following glucose addition in the same manner as for *PCK1* mRNA (data not shown). However, the protein was stable in glucose along the time course of the experiment. This result indicates that the addition of glucose triggers the dephosphorylation of Cat8p and induces the repression of the corresponding mRNA. However, this seems not to be a signalling for proteolytic degradation, as we demonstrated by immunoblot detection using gradient SDS-PAGE. In these experiments, we have not found any degradation products of Cat8p after the addition of glucose in the time course studied (data not shown).

GLC7 (*CID1*) encodes a protein phosphatase necessary for glucose repression, and its function was hypothesized to be antagonistic to the Cat1p protein kinase (53). To determine its possible function in the glucose regulation of gluconeogenic enzymes, we investigated the kinetics of Cat8pIII dephosphorylation in a *glc7* mutant after glucose addition to derepressed cells. However, the dephosphorylation pattern of Cat8pIII (Fig. 7) and the enzymatic inactivation of PEPCK were identical to those of wild-type cells (data not shown). This result shows that Glc7p is not the protein phosphatase responsible for Cat8pIII dephosphorylation, indicating the presence of some other protein phosphatase(s) involved in this glucose signalling pathway.

DISCUSSION

CAT8 was previously identified as an essential gene for gluconeogenic and glyoxylate cycle enzyme derepression (20, 40). In this study, we identified in the upstream region of *CAT8* a 20-bp *cis*-regulatory element, URE_{CAT8}, which comprises a sequence motif for the negative regulator Mig1p. Deletion of the Mig1p-binding site in a *CAT8-lacZ* fusion resulted in a total loss of derepression. In agreement with that finding, promoter fragments which lack the Mig1p-binding motif were not able to mediate transcriptional activation after insertion into a UAS-less heterologous promoter. Previously we could show genetically that Mig1p mediates glucose repression of *CAT8* (20). The loss of glucose repression in assays using a *CAT8-lacZ* reporter in a *mig1* deletion ruled out an activating function for Mig1p itself. Recently the identification of a Mig1p homolog, Mig2p, which can bind to Mig1p recognition sites and acts mainly as a repressor on glucose has been reported (29). The *mig2* deletion showed no phenotype concerning the regulation of gluconeogenic genes. Because of the lower binding affinity, an important role for Mig2p in *CAT8* regulation is less likely. However, as *CAT8* expression was not assayed in that study, binding of Mig2p to the *CAT8* promoter cannot be ruled out and needs to be tested in future experiments.

In this study, we demonstrated the regulatory properties of a 20-bp URE_{CAT8} containing the Mig1p-binding site after its insertion into a UAS-less heterologous promoter. The data clearly confirmed a negative regulatory function of URE_{CAT8} under repressing conditions and an activating function under derepressing conditions.

The transcription of *CAT8*, however, is not sufficient for derepression of gluconeogenic genes. This becomes obvious in a *mig1* mutant grown on glucose, where *CAT8* is expressed but the gluconeogenic genes remain repressed. From this genetic evidence, we concluded that Cat8p should be posttranslationally activated.

In this work, we show that Cat8p is specifically phosphorylated in response to the release from glucose repression. Multiple phosphorylated forms were also found for other transcriptional activators such as Gal4p (32, 33), Abf1p (49), and Adrlp (7). It was suggested for these proteins that the different phosphorylation states drive the function of these proteins to different target genes. Recently it was reported that Gal4p is phosphorylated in the nucleus, as a consequence of its transcriptional activating function (42). Putative protein kinase genes involved in this process could be those encoding nuclear cyclin-dependent protein kinases, which are subunits of the RNA polymerase II holoenzyme (*KIN28* [6]), proteins in the mediator complex (*SSN3-SSN8* [2, 24]), or even other transient regulatory elements (*CTKD-I* [50]).

Interestingly, a $Gal4_{BD}$-Cat8 protein was still carbon source regulated. The fact that the hybrid activator is not functional and appears only partially phosphorylated in a *cat1* deletion strain suggested that Cat1p is necessary for the activation of Cat8p by phosphorylation. However, two-hybrid studies and coimmunoprecipitation failed to prove that Cat8p is a direct phosphorylation target of the Cat1 protein kinase (data not shown). Nevertheless, the possible interaction between Cat1p and Cat8p may be transient and probably also needs other structural components of the Cat1 kinase multimer, such as Cat3p and Sip1p. To examine a possible indirect role of Cat1p via Mig1p on posttranslational modifications of Cat8p, we compared the phosphorylation patterns of Cat8p in a *cat1* mutant and in the double-mutant *mig1 cat1* strain. Both strains showed Cat8pII, indicating that another Cat1p-Mig1p-independent protein kinase is needed for this modification. However, this phosphorylation is not sufficient to activate Cat8p to function as a transcriptional activator for gluconeogenic genes. The Cat8pIII form was detectable in a *mig1 cat1* mutant, but we could not rule out the possibility that another protein kinase phosphorylates the overexpressed Cat8p unspecifically by kinase cross talk.

The ethanol-specific Cat8p modification preceded the induction of the *PCK1* mRNA. Several experimental data suggest that other transcriptional factors, expressed in response to

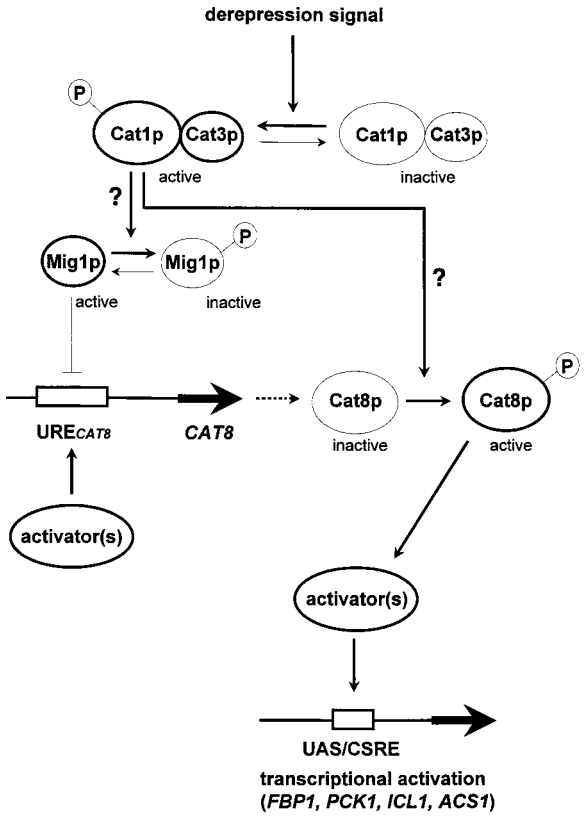

FIG. 8. Model of gluconeogenic gene regulation.

Cat8pIII, are necessary for gluconeogenic derepression: we observed a lag period (about 1 h) between Cat8p phosphorylation and the induction of *PCK1* mRNA, and the derepression of gluconeogenic target genes was dependent on de novo protein synthesis even when Cat8pIII was present. In gel retardation experiments, two UAS_{PCK1}-protein complexes were detected in derepressed cell extracts (39), and its presence was dependent on *CAT1* and *CAT8*. Nevertheless, none of these complexes was influenced by using Cat8p-specific antiserum or by *CAT8* overexpression (37a). Similar approaches to detect a direct Cat8p binding also failed for the *ICL1* promoter (40). In contrast to our previous hypothesis, these results suggest that Cat8p is not the protein directly binding to the gluconeogenic UAS elements but could activate the putative transcriptional factors detected in the DNA-protein complexes. Surprisingly, these factors were not identified by the screening used to isolate *CAT8*. However, the selection system also failed to obtain known regulatory factors in gluconeogenesis, like *CAT1* or *CAT3*, probably because of the restricted conditions used. The genetic approach may also have failed because isofunctional activators mediate gluconeogenic derepression. Alternative strategies have been used to identify these unknown elements by multicopy suppression of *cat8*, but they also failed to isolate other regulators (37a).

Cat8p, as a key activator element in the gluconeogenesis, is susceptible to the presence of glucose in the medium. The kinetics of Cat8pIII dephosphorylation after glucose addition are similar to those found for Gal4p (32) and Abf1p (49). However, for other transcriptional factors (Adr1p), glucose could trigger inactivation by phosphorylation (7). As shown by the normal Cat8pIII dephosphorylation in *glc7* (*cid1*) mutants, the Glc7p protein phosphatase is not involved in the dephosphorylation of Cat8p. This finding suggests that another protein phosphatase is active in the presence of glucose.

From the results presented, the following model for glucose derepression of gluconeogenic genes can be derived (Fig. 8). In the presence of glucose, the Mig1p repressor binds to the URE_{CAT8} element of the *CAT8* promoter and prevents its transcription. Without glucose, the Cat1p-Cat3p kinase becomes active, phosphorylates Mig1p, and prevents its binding to *CAT8*. However, *CAT8* expression needs the binding of a still unknown transcriptional activator to URE_{CAT8}. This binding motif largely overlaps or is even identical with the Mig1p-binding site. Thereafter, the synthesized Cat8p is phosphorylated to Cat8pIII in a Cat1p-Cat3p-dependent manner. Cat8pIII in turn probably activates the expression of other transcriptional factors which finally derepress the gluconeogenic genes upon binding to the respective UAS elements.

ACKNOWLEDGMENTS

We thank M. J. Lafuente, J. Haase, and A. Hofmann for helpful collaboration and Al Plummer for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft and the MEC of Spain.

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