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Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes

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The carbon source-responsive element (CSRE) mediates transcriptional activation of the gluconeogenic genes during growth of the yeast *Saccharomyces cerevisiae* on non-fermentable carbon sources. Previous studies have suggested that the Cat8 protein activates the expression of CSRE-binding factors. We show here that one of these factors is Sip4, a glucose-regulated C_6 zinc cluster activator which was identified by its interaction with the Snf1 protein kinase. We present genetic evidence that Sip4 contributes to transcriptional activation by the CSRE and biochemical evidence that Sip4 binds to the CSRE. Binding was detected in electrophoretic mobility shift assays with both yeast nuclear extracts and a bacterially expressed Sip4 fusion protein. Evidence suggests that Sip4 also activates the expression of other CSRE-binding proteins. Finally, we show that Cat8 regulates *SIP4* expression and that overexpression of Sip4 compensates for loss of Cat8. We propose a model for activation by the CSRE in which Sip4 and Cat8 have related functions, but Cat8 is the primary regulator because it controls Sip4 expression.

Keywords: carbon source-responsive element/Cat8/gluconeogenesis/*Saccharomyces cerevisiae*/Sip4

Introduction

The carbon source-responsive element (CSRE) is required to activate transcription of the genes of the gluconeogenic pathway and the glyoxylate cycle in the yeast *Saccharomyces cerevisiae*. This regulatory element is present in the promoter regions of the genes *ICL1*, *FBP1*, *PCK1*, *MLS1* and *ACS1* (Niederacher *et al*., 1992; Schöler and Schüller, 1994; Hedges *et al*., 1995; Proft *et al*., 1995a; Vincent and Gancedo, 1995; Caspary *et al*., 1997; Kratzer and Schüller, 1997). These genes are expressed during growth on non-fermentable carbon sources, such as ethanol or acetate, and repressed during growth on glucose (for review see Gancedo, 1998). Accordingly, activation by the CSRE shows similar carbon source regulation and depends on the Snf1 (Cat1, Ccr1) protein kinase (Schöler and Schüller, 1994; Hedges *et al*., 1995; Proft *et al*., 1995b; Vincent and Gancedo, 1995), which has a central role in regulating carbon source utilization (Celenza and Carlson, 1986; Schüller and Entian, 1987).

Gene activation mediated by the CSRE also requires the *CAT8* (*DIL1*) gene, which encodes a transcriptional activator with a C_6 zinc cluster motif characteristic of DNA-binding proteins in the Gal4 family (Hedges *et al*., 1995; Rahner *et al*., 1996). Both the expression of *CAT8* and the activator function of the protein are regulated by glucose and are dependent on Snf1; moreover, Cat8 is phosphorylated in response to glucose limitation, and some of the phosphorylations depend on Snf1 (Hedges *et al*., 1995; Rahner *et al*., 1996; Randez-Gil *et al*., 1997). These findings implicate Cat8 in the carbon source- and Snf1-dependent activation by the CSRE. However, evidence suggests that Cat8 does not bind directly to this element but rather activates the expression of other proteins that are, in turn, the direct activators of the CSRE (Rahner *et al*., 1996; Randez-Gil *et al*., 1997).

Here, we have examined the role of Sip4, another C_6 zinc cluster protein, in activation by the CSRE. Sip4 was identified in a two-hybrid screen as a protein which interacts with the Snf1 kinase and was shown to function as a transcriptional activator (Yang *et al*., 1992; Lesage *et al*., 1996). The expression of the *SIP4* gene is regulated by glucose, and the activation of a reporter by a constitutively expressed LexA–Sip4 fusion protein is inhibited by glucose and dependent on the Snf1 kinase. Snf1 is also required for the differential phosphorylation of Sip4 in response to glucose availability. These findings strongly suggest that the Snf1 kinase interacts with Sip4 to modulate its activity, but the genes activated by Sip4 remained unknown because no phenotype was detected in a *sip4*Δ mutant.

Several lines of evidence have suggested a connection between Sip4 and CSRE function. First, the activation functions of Sip4 and the CSRE are similarly regulated by carbon source and the Snf1 kinase. Secondly, a new consensus for the CSRE (Caspary *et al*., 1997) matches a sequence in the *SIP4* promoter, suggesting that expression of *SIP4* and the gluconeogenic genes are also similarly regulated. Finally, DNA microarray analysis of gene expression during the diauxic shift (DeRisi *et al*., 1997) showed that *SIP4* exhibits the same expression profile as the gluconeogenic genes; moreover, *SIP4* was found to be one of only two transcription factor genes (the other is *HAP4*) that are induced substantially in parallel with the gluconeogenic genes upon depletion of glucose.

In this study, we show that Sip4 is required for maximal activation by the CSRE and that Sip4 is a CSRE-binding factor. We present evidence that Sip4 affects activation by the CSRE not only by binding to this sequence but also by activating the expression of other CSRE-binding proteins. Finally, we show that *SIP4* expression requires Cat8 and that overexpression of Sip4 compensates for loss of Cat8.

Results

Sip4 is required for maximal transcriptional activation by the CSRE

To determine whether Sip4 affects the regulation of the CSRE, we first constructed a CSRE–*lacZ* reporter containing the *CYC1* core promoter fused to *lacZ* under the control of the CSRE from the *FBP1* (fructose-1,6-bisphosphatase gene) promoter. The sequence of this CSRE matches the consensus for the strong CSRE (CCRTYCRTCCG; R = A or G, Y = C or T) (Rahner *et al*., 1996). In a wild-type strain, this reporter is repressed when cells are grown in high glucose and activated after a shift to low glucose or during growth in non-fermentable carbon sources (Figure 1), which is consistent with the known properties of the CSRE. We then followed the kinetics of activation of this reporter in the wild-type and an isogenic *sip4*Δ deletion mutant after a shift from high to low glucose. Both strains started to derepress expression of the reporter after 2 h, but the maximal level of β-galactosidase was 2-fold lower in the mutant than in the wild-type (Figure 1A). In an independent experiment, strains were shifted to glycerol plus ethanol; in this case, the reporter was derepressed to higher levels, but activity was again almost 2-fold lower in the mutant than in the wild-type (Figure 1B). These results indicate that Sip4 is responsible for half of the transcriptional activation conferred by the CSRE.

Expression of GAD–Sip4 activates the CSRE in glucose-grown cells

To confirm that Sip4 is involved in the regulation of the CSRE, we took advantage of the fact that the CSRE is normally repressed in glucose-grown cells. The expression of *SIP4* is also glucose-repressed, and its transcriptional activation function is inhibited by glucose (Lesage *et al*., 1996). To circumvent the glucose regulation of Sip4 function, we expressed a fusion of the Gal4 activation domain (GAD) to Sip4 from the *ADH1* promoter. We then tested whether expression of GAD–Sip4 activates the CSRE–*lacZ* reporter in glucose-grown cells. β-Galactosidase activity was 800-fold higher in cells expressing GAD–Sip4 than in control cells expressing only GAD (Figure 1C). This result indicates that Sip4 functions, either directly or indirectly, to activate the CSRE.

Galactose inhibits the function of both the CSRE and Sip4

The CSRE is negatively regulated not only by glucose but also by other fermentable carbon sources such as galactose (Schöler and Schüller, 1994). To test whether the transcriptional activation function of Sip4 is inhibited by galactose, we assayed the ability of LexA–Sip4 (expressed from pLexA$_{202}$–SIP4) to activate a *lexAop–lacZ* reporter [p1840; identical to 1145 (Brent and Ptashne, 1985)] in wild-type strain FY250. β-galactosidase activity was low during growth in 2% galactose (3.7 ± 0.4 U; values are averages for four transformants); for comparison, activity was equivalently low during growth in 2% glucose (2.8 ± 0.6 U) and increased substantially during a shift to 0.05% glucose (39.7 ± 1.9 U after 3 h). Thus, Sip4 does not activate transcription effectively in the presence of galactose, which is consistent with the lack of CSRE function.

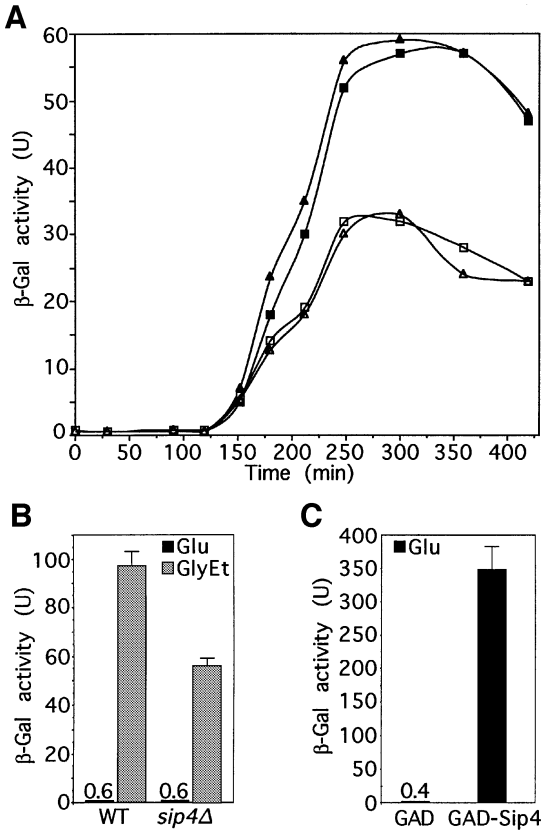

Fig. 1. Sip4 activates the CSRE. (**A**) Wild-type W303–1A (solid symbols) and an isogenic *sip4*Δ mutant MCY4026 (open symbols) were transformed with pOV22, carrying the CSRE–*lacZ* reporter. Two different transformants of each strain were grown selectively to mid-log phase in 2% glucose and then shifted (t = 0) to 0.05% glucose. Samples were taken at the indicated times and β-galactosidase activity was assayed. Control transformants carrying the parental vector (pΔSS) gave no β-galactosidase activity (<1 U) under these conditions. (**B**) Wild-type (WT) MCY4031 and *sip4*Δ mutant MCY4026 were transformed with pOV22. Transformants were grown in 2% glucose (Glu) and shifted to 2% glycerol + 2% ethanol (GlyEt) for 5 h 30 min. Both strains expressed maximal levels of β-galactosidase activity at 5 h 30 min, as determined by an analysis similar to that shown in (A). Values are averages for four transformants, and standard deviations are indicated. MCY4031 carrying the vector pΔSS expressed no β-galactosidase activity (<1 U) in GlyEt. (**C**) Wild-type FY250 was cotransformed with the reporter pOV22 and with the multicopy plasmid pGAD–SIP4 or the vector pACTII, expressing GAD–Sip4 and GAD, respectively. Transformants were grown to mid-log phase in 2% glucose. Values are average β-galactosidase activity for four transformants, and standard deviations are indicated. FY250 carrying the vector pΔSS and pGAD–Sip4 expressed no β-galactosidase activity (<1 U) under these conditions.

Sip4 is involved in the formation of DNA–protein complexes with the CSRE in vitro

The CSRE forms specific DNA–protein complexes in an electrophoretic mobility shift assay (EMSA) with crude protein extracts. Some of these complexes form only with extracts from derepressed wild-type cells and not with extracts from glucose-repressed cells; moreover, formation of these complexes is dependent on *SNF1* (*CAT1*) or *CAT8* (Niederacher *et al*., 1992; Schöler and Schüller, 1994; Proft *et al*., 1995b; Rahner *et al*., 1996; Caspary *et al*., 1997; Kratzer and Schüller, 1997). When nuclear

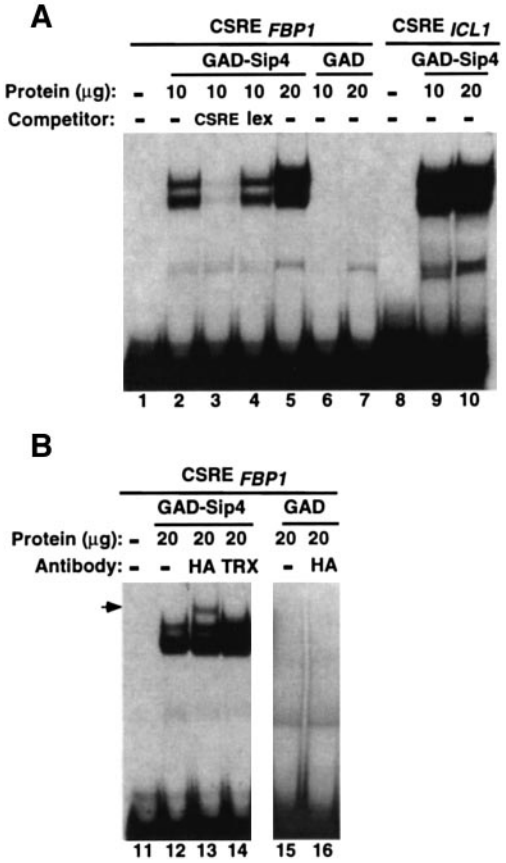

Fig. 2. Expression of GAD–Sip4 causes formation of complexes with the CSRE in extracts from glucose-grown cells. Nuclear extracts were prepared from glucose-grown cultures of strain W303-1A transformed with pGAD–SIP4 or the vector pACTII. The $CSRE_{FBP1}$ and $CSRE_{ICL1}$ oligonucleotides (OL1 and OAS12, respectively) were ^{32}P-labeled and incubated with nuclear extracts as described in Materials and methods. Samples in lanes 1, 8 and 11 contained no added protein, and samples in lanes 2–10 and 12–16 contained 10 or 20 μg of nuclear protein, as indicated. (**A**) Unlabeled $CSRE_{FBP1}$ oligonucleotide (specific competitor; lane 3) or lex oligonucleotide (non-specific competitor; lane 4) was added in 100-fold excess relative to the labeled probe. (**B**) Monoclonal antibodies (40 ng) against HA (Boehringer Mannheim) or TRX (Santa Cruz Biotechnology) were added to the nuclear extracts and incubated for 5 min before addition of the oligonucleotide probe. The supershifted band in lane 13 is indicated with an arrow.

extracts are used, only the carbon source- and *SNF1*-dependent complexes are detected (Vincent and Gancedo, 1995).

To assess the effect of Sip4 on the formation of such complexes, we examined whether the strong activation of the CSRE in glucose-grown cells expressing GAD–Sip4 correlates with the formation of specific complexes with the CSRE. We prepared nuclear extracts from glucose-grown cells expressing GAD–Sip4 or GAD from the *ADH1* promoter. Extracts were incubated with a ^{32}P-labeled oligonucleotide containing the CSRE sequence from the *FBP1* promoter, designated $CSRE_{FBP1}$, and DNA–protein complexes were resolved by electrophoresis on a native polyacrylamide gel (Figure 2A). Two specific complexes were formed when extracts were prepared from cells expressing GAD–Sip4 (lanes 2–5), whereas no complexes were detected when only GAD was expressed (lanes 6–7). Formation of these complexes was effectively competed by addition of excess unlabeled $CSRE_{FBP1}$ oligonucleotide (lane 3) but not by an unrelated oligonucleotide containing the *Escherichia coli* LexA operator (lane 4), indicating that these complexes reflect specific binding to the CSRE. Complexes were also detected when extracts were incubated with another CSRE derived from the *ICL1* (isocitrate lyase gene) promoter, designated $CSRE_{ICL1}$ (lanes 8–10). The formation of these complexes by extracts from glucose-grown cells expressing GAD–Sip4 confirms the genetic evidence that Sip4 either binds the CSRE or regulates other CSRE-binding proteins.

To test whether GAD–Sip4 is present in these complexes, we used a monoclonal anti-hemagglutinin (HA) antibody that recognizes an HA epitope present in GAD–Sip4. Addition of the HA antibody to the nuclear extract prior to incubation with DNA resulted in the appearance of a new band of lower mobility (Figure 2B; lane 13). In control experiments, no supershift was observed with an unrelated antibody to thioredoxin (TRX) (lane 14) or with antibodies to LexA or the Gal4 DNA-binding domain (data not shown), and addition of HA antibody to extracts containing only GAD did not produce such a band (lane 16). These results indicate that GAD–Sip4 is a component of the DNA–protein complexes formed with the CSRE.

However, the addition of HA antibody did not affect the mobility of the bulk of the complexes. Use of another HA antibody preparation gave similar results, as did experiments with extracts containing Sip4 tagged with a triple HA epitope (data not shown). These results suggest that Sip4 is present in a minor fraction of the complexes and that most of the complexes contain another factor(s) which binds this sequence. Thus, expression of GAD–Sip4 in glucose-grown cells may activate the expression of other CSRE-binding proteins.

Bacterially expressed $Sip4_{1–130}$ binds to the CSRE

To confirm that Sip4 binds directly to the CSRE, we expressed in *E.coli* a glutathione *S*-transferase (GST) fusion to the Sip4 DNA-binding domain (residues 1–130), designated GST–$Sip4_{1–130}$. This region of Sip4 contains the C_6 zinc cluster DNA-binding motif, the linker residues, and the predicted coiled-coil dimerization domain (Lesage *et al.*, 1996). This region is sufficient for recognition of the CSRE because the analogous GAD fusion (GAD–$Sip4_{1–130}$; pOV30) strongly activates the CSRE–*lacZ* reporter (data not shown). GST–$Sip4_{1–130}$ was purified from bacterial extracts by binding to glutathione–agarose beads and eluting with free glutathione. The purified fusion protein was incubated with ^{32}P-labeled oligonucleotide $CSRE_{FBP1}$, and DNA–protein complexes were resolved by electrophophoresis in a native polyacrylamide gel (Figure 3). GST–$Sip4_{1–130}$ bound specifically to the $CSRE_{FBP1}$; binding was competed by addition of unlabeled CSRE oligonucleotide but not by addition of an oligonucleotide containing the LexA operator (lanes 3 and 4). GST–$Sip4_{1–130}$ also recognized $CSRE_{ICL1}$ (lanes 10 and 11). Purified GST alone did not bind to the CSRE (lanes 6 and 7). These results demonstrate that Sip4 is a CSRE-binding factor.

Overexpression of Sip4 compensates for loss of Cat8

A *cat8*Δ mutant is defective for CSRE activation and unable to grow on non-fermentable carbon sources

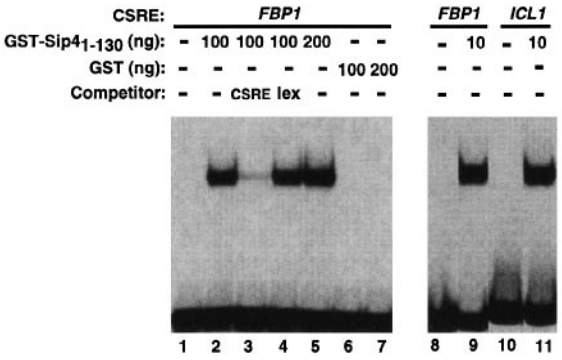

Fig. 3. Bacterially expressed Sip4 DNA-binding domain binds to the CSRE sequence. Purified, bacterially expressed GST–Sip4$_{1–130}$ and GST proteins were incubated with ^{32}P-labeled CSRE$_{FBP1}$ and CSRE$_{ICL1}$ oligonucleotides as described in Materials and methods, except that no poly(dI–dC) was added in lanes 8–11. Samples in lanes 1, 8 and 10 contained no added protein, and samples in lanes 2–7, 9 and 11 contained the indicated amount of GST or GST–Sip4$_{1–130}$. Less protein was required to give a band shift in the absence of poly(dI–dC) (lanes 8–11). Unlabeled CSRE$_{FBP1}$ oligonucleotide (specific competitor; lane 3) or lex oligonucleotide (non-specific competitor; lane 4) was added in 100-fold excess relative to the labeled probe.

(Hedges *et al*., 1995; Rahner *et al*., 1996). Since Sip4 is involved in the activation of the CSRE, we examined the functional relationship of Sip4 and Cat8. First, we tested whether overexpression of Sip4 from the *ADH1* promoter on pOV42 restores activation of the CSRE–*lacZ* reporter in a *cat8*Δ mutant. Transformants carrying the reporter and either pOV42 or the parental vector were grown in glucose and shifted to glycerol plus ethanol for 5 h 30 min. β-galactosidase activity was 29-fold higher (25.9 U) in derepressed mutant cells overexpressing Sip4 than in cells carrying the vector (0.9 U) (Figure 4A). Thus, overexpression of Sip4 compensates partially for the loss of Cat8 in activation of the CSRE$_{FBP1}$. In addition, overexpression of Sip4 partially bypassed the glucose regulation of the reporter in both wild-type and mutant cells.

We next examined whether overexpression of Sip4 also restores growth of a *cat8*Δ mutant on non-fermentable carbon sources. We transformed wild-type and *cat8*Δ mutant strains in two different genetic backgrounds (W303 and FY250) with pOV29, which expresses Sip4 from the *ADH1* promoter, or the parental vector. The *cat8*Δ mutant transformants carrying pOV29 were able to grow on synthetic medium containing 3% ethanol, indicating that overexpression of Sip4 compensates for the lack of Cat8 (Figure 5); all transformants grew well on 2% glucose (data not shown). This result suggests that Sip4 not only activates the CSRE$_{FBP1}$ but also more generally activates the expression of the genes that are controlled by the CSRE and are required for growth on non-fermentable carbon sources.

Regulation of SIP4 expression by Sip4 and Cat8

The *SIP4* promoter contains a sequence (CCGTTCG-ACCG) that matches the less stringent consensus for the CSRE (CCRTYSRNCCG; S = C or G) (Caspary *et al*., 1997), and previous work showed that the expression of *SIP4* is glucose-repressed and dependent on the Snf1 kinase (Lesage *et al*., 1996). These findings suggested that the expression of *SIP4* may either be regulated by Cat8

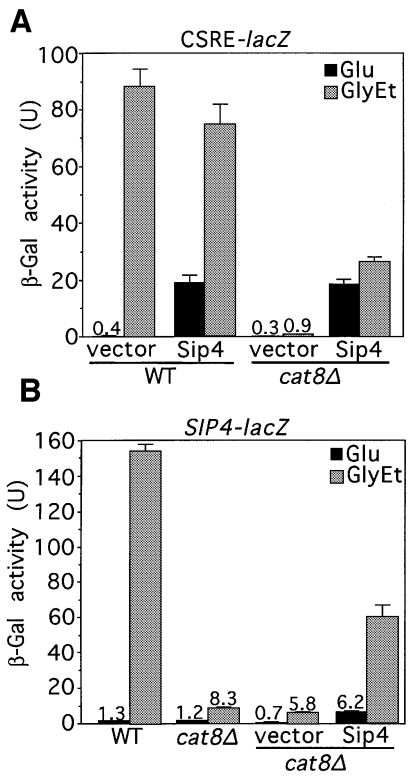

Fig. 4. Overexpression of Sip4 restores activation of CSRE–*lacZ* and *SIP4–lacZ* in a *cat8*Δ mutant. Isogenic strains W303-1A (wild-type, WT) and MCY4029 (*cat8*Δ) were cotransformed with the multicopy plasmid pOV42, expressing Sip4 from the *ADH1* promoter, or the vector pSK134 and a plasmid carrying the reporter (**A**) CSRE–*lacZ* (pOV22) or (**B**) *SIP4–lacZ* (pSIP4–lacZ). Transformants were grown in 2% glucose (Glu) and shifted to 2% glycerol + 2% ethanol (GlyEt) for 5 h 30 min. Values are average β-galactosidase activity for four transformants, and standard deviations are indicated. The increase in *SIP4–lacZ* expression during derepression (100-fold) is greater than that reported previously (10-fold) after a shift to 0.05% glucose for 3 h (Lesage *et al*., 1996).

or autoregulated. To assess the first possibility, we measured the expression of a *SIP4–lacZ* fusion containing 1170 bp of the promoter and 51 bp of the coding sequence (Lesage *et al*., 1996) in wild-type and *cat8*Δ mutant strains after a shift to ethanol plus glycerol (Figure 4B). β-galactosidase activity was 18-fold lower in the mutant than in the wild-type (8.4 and 153 U, respectively), showing that Sip4 expression is controlled by Cat8.

To address the possibility of autoregulation, we first compared expression of the *SIP4–lacZ* fusion in wild-type and *sip4*Δ mutant strains after a shift to ethanol plus glycerol, as above; however, expression was not significantly different in the two strains (data not shown). We then tested whether overexpression of Sip4 from the *ADH1* promoter on pOV42 restores the activation of *SIP4–lacZ* in a *cat8*Δ mutant. β-galactosidase activity was increased 10-fold in derepressed cells overexpressing Sip4 relative to those carrying the vector (60 and 5.8 U, respectively) (Figure 4B). These results show that when overexpressed, Sip4 is able to activate its own expression.

Discussion

Expression of the gluconeogenic genes is regulated by the CSRE promoter elements in response to carbon source.

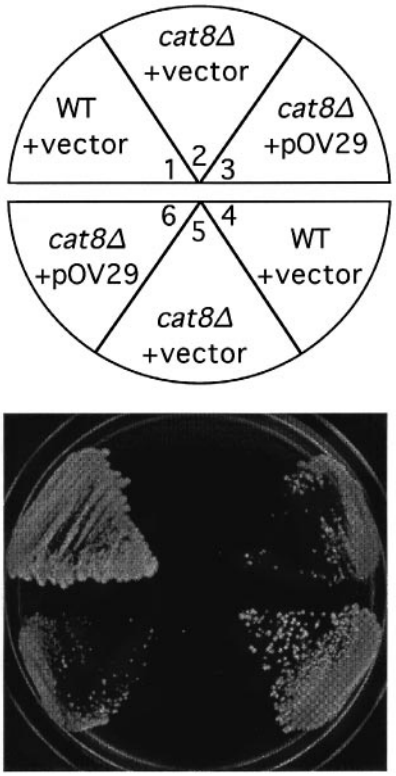

Fig. 5. Overexpression of Sip4 restores growth of *cat8*Δ mutants on ethanol. Strains were (1–3) W303-1A (wild-type, WT) and the isogenic disruptant MCY4029 (*cat8*Δ) and (4–6) FY250 (WT) and the isogenic strain MCY4030 (*cat8*Δ). Strains were transformed with the multicopy plasmid pOV29, expressing Sip4 from the *ADH1* promoter, or the vector pWS93. Transformants were streaked on selective SC–Ura +3% ethanol. Plates were incubated at 30°C for 7 days and photographed.

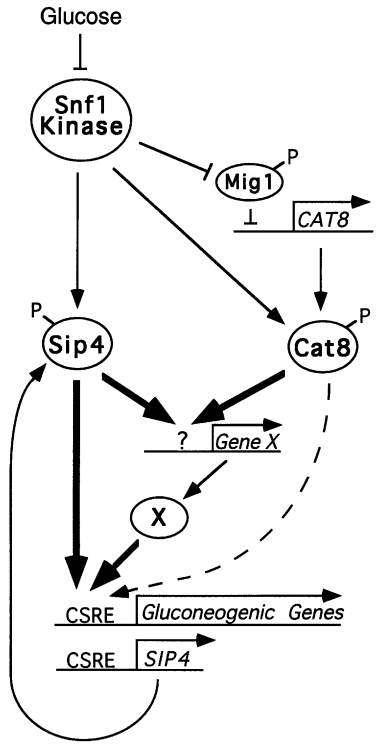

Fig. 6. Model for the role of Sip4 in CSRE-mediated activation. The model is described in the text. Heavy arrows indicate DNA binding of transcriptional activators to the CSRE and to the promoters of the proposed gene(s) (*Gene X*) encoding unidentified CSRE-binding activators (X). The dashed arrow indicates possible direct binding of Cat8 to the CSRE. The role of the Snf1 kinase in activating Sip4 and Cat8 is indicated, as is its role in inhibiting Mig1 repressor function via phosphorylation (Ostling and Ronne, 1998; Treitel *et al*., 1998). Expression of *CAT8* is regulated by Mig1 (Hedges *et al*., 1995; Rahner *et al*., 1996; Randez-Gil *et al*., 1997); no Mig1 site was found in the promoter of *SIP4*.

Previous work showed that CSRE activation requires Cat8, a C_6 zinc cluster activator protein, but no binding of Cat8 to the CSRE has been reported. Sip4 is another C_6 zinc cluster activator that shows the same regulation by carbon source and Snf1 kinase as does the CSRE. We show here that Sip4 affects CSRE function *in vivo* and binds directly to the CSRE *in vitro*. We further show that expression of *SIP4* is under the control of Cat8 and is probably also subject to autoregulation.

We present four lines of genetic evidence that implicate Sip4 in the activation of the CSRE. First, activation of the CSRE is reduced 2-fold in *sip4*Δ mutants**.** Secondly, expression of GAD–Sip4 from the constitutive *ADH1* promoter, a strategy that bypasses the glucose regulation of Sip4 function, activates the CSRE in glucose-grown cells. Thirdly, we show that the activation function of LexA–Sip4 is inhibited not only by glucose but also by galactose, as is the CSRE. Finally, although activation of the CSRE is more stringently dependent on Cat8 than on Sip4, overexpression of Sip4 compensates for loss of Cat8. In a *cat8*Δ mutant, overexpression of Sip4 restores activation of the CSRE–*lacZ* reporter and also restores growth on ethanol. These findings indicate that Sip4 functions, either directly or indirectly, to activate the CSRE.

We further show that Sip4 is involved in the formation of DNA–protein complexes with the CSRE *in vitro*. Previous EMSA studies showed that the CSRE forms specific DNA–protein complexes when incubated with nuclear extracts but only when the extracts are prepared from derepressed cells (Vincent and Gancedo, 1995). We show that expression of GAD–Sip4 from the *ADH1* promoter allows formation of two specific DNA–protein complexes with nuclear extracts from glucose-repressed cells, indicating that GAD–Sip4 binds the CSRE or regulates other CSRE-binding proteins. The supershifted mobility of a minor fraction of the complexes upon addition of a specific antibody indicates that GAD–Sip4 is a component of at least some of the DNA–protein complexes formed with the CSRE. Finally, to confirm that Sip4 binds directly to the CSRE, we show that a bacterially expressed GST fusion to the Sip4 DNA-binding domain binds to the CSRE *in vitro*.

Sip4 is an unusual member of the C_6 zinc cluster DNA-binding protein family in that the CSRE consensus contains two triplets, CGG and T/CGG, in a direct orientation separated by five base pairs. The binding sites for most members of this family contain two separated CGG triplets in inverted orientation. Hap1 and Mal63 are the only other family members known to bind a site with the triplets positioned as direct repeats (Zhang and Guarente, 1994; Sirenko *et al*., 1995). The *Aspergillus nidulans* FacB protein, which activates acetate utilization genes, is highly similar to Sip4 in the C_6 zinc cluster DNA-binding motif and linker region, which determines the spacing between

the triplets of the recognition site (Reece and Ptashne, 1993); the proposed consensus recognition sites do not resemble the CSRE (Todd *et al*., 1998), but five of the footprinted sites also contain CGG and NGG triplets separated by 5 bp.

The EMSA results suggest that Sip4 not only binds the CSRE but also activates the expression of other CSRE-binding protein(s) (designated X in Figure 6). Most of the DNA–protein complexes resulting from overexpression of GAD–Sip4 appear to contain another factor(s). This factor is not the endogenous Sip4 because GAD–Sip4 does not significantly activate a *SIP4–lacZ* reporter in glucose-grown cells (data not shown). Cat8 is also an unlikely candidate because GAD–Sip4 confers CSRE–*lacZ* expression in glucose-grown cells independently of Cat8 (data not shown). Thus, expression of GAD–Sip4 in glucose-grown cells probably activates the expression of genes encoding other CSRE-binding proteins. Such genes may contain a CSRE-like sequence in their promoter, or Sip4 may recognize other sites; previous studies demonstrated that a C_6 zinc cluster protein may bind different sequences (Pfeifer *et al*., 1987).

Previous studies have proposed that Cat8 activates the expression of genes encoding CSRE-binding factors (Rahner *et al*., 1996; Randez-Gil *et al*., 1997), and we present evidence here that Sip4 is one such factor. Expression of *SIP4–lacZ* is strongly decreased in a *cat8*Δ mutant, indicating that Cat8 regulates the transcription of *SIP4*. This finding is consistent with evidence that expression of Sip4 from its native chromosomal locus is not sufficient to allow the growth of a *cat8*Δ mutant on ethanol, whereas overexpression of Sip4 from the *ADH1* promoter restores growth of the mutant.

Sip4 cannot be the only CSRE-binding factor that is absent in a *cat8*Δ mutant. CSRE-mediated activation is nearly abolished in a *cat8*Δ mutant and reduced only 2-fold in a *sip4*Δ mutant. Cat8 may directly activate the expression of other CSRE-binding proteins besides Sip4, possibly the same proteins that are activated by Sip4 (Figure 6). We also suggest that Cat8 itself binds to the CSRE. Cat8 shows high similarity to Sip4, not only in the C_6 zinc cluster DNA-binding motif but also within the linker region, suggesting that Cat8 has the same DNA-binding specificity as Sip4; however, no such binding has been detected (Rahner *et al*., 1996; Randez-Gil *et al*., 1997).

We propose a model for activation by the CSRE in which Sip4 and Cat8 have related functions, but Cat8 is the primary regulator because it controls Sip4 expression (Figure 6). During growth on non-fermentable carbon sources, the Snf1 kinase inhibits transcriptional repression of the *CAT8* gene by the Mig1 repressor and upregulates the transcriptional activator function of the Cat8 protein, as proposed previously (Rahner *et al*., 1996; Randez-Gil *et al*., 1997). Cat8 then activates the expression of the CSRE-regulated genes, including *SIP4*, by activating the expression of genes encoding CSRE-binding proteins (Figure 6, X) or possibly by binding to the CSRE. The transcriptional activator function of Sip4 is in turn upregulated by Snf1, and Sip4 participates in activation of the CSRE-regulated genes. Sip4 binds directly to the CSRE and also activates the expression of the other CSRE-binding factors.

This model is consistent with evidence that Sip4 and Cat8 are closely related in function. Moreover, it accounts for the more severe phenotype caused by a *cat8*Δ mutation because transcription of *SIP4* requires Cat8. Finally, this model is compatible with the idea that Sip4 and Cat8 both recognize the CSRE but also can accommodate different DNA-binding specificities for the two proteins.

Materials and methods

Strains and genetic methods

Saccharomyces cerevisiae strains were FY250 (*MAT*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52*); MCY4030 (FY250 *cat8*Δ*::TRP1*); and W303–1A (*MAT***a** *trp1-1 leu2-3,112 his3-11,15 ura3-1 ade2-1 can1-100*) and its isogenic derivatives MCY4026 (*sip4*Δ*::LEU2*), MCY4029 (*cat8*Δ*::TRP1*) and MCY4031 (*LEU2* restored). *SIP4* was disrupted using a fragment from plasmid pPL41 (gift of P.Lesage) containing the *SIP4* gene with codons 51–2067 replaced by *LEU2*. *CAT8* disruptions were made using plasmid pOV38 containing *CAT8* with codons 838–3351 replaced by *TRP1*. Standard genetic methods were followed, and yeast cultures were grown in synthetic complete (SC) medium lacking appropriate amino acids to maintain selection for plasmids (Rose *et al*., 1990).

Oligonucleotides

OL1 contains the $CSRE_{FBP1}$ and was obtained by mixing equimolar amounts of oligonucleotides 5′-TCGACTTCCGGACGGATGGAATCG-3′ and 5′-TCGACGATTCCATCCGTCCGGAAG-3′ (Vincent and Gancedo, 1995). OAS12 contains the $CSRE_{ICL1}$ and was obtained by mixing equimolar amounts of oligonucleotides 5′-TCGAGTTTCCATT-CATCCGAGCGAGATCT-3′ and 5′-TCGAAGATCTCGCTCGGATG-AATGGAAAC-3′ (Schöler and Schüller, 1994). The lex oligonucleotide is a double-stranded palindromic sequence containing the LexA operator, 5′-CCGGTACTGTATGTACATACAGTA-3′.

Plasmids

pGAD–SIP4 and pSIP4–lacZ were as described previously (Lesage *et al*., 1996). $pLexA_{202}$–SIP4 (gift of P.Lesage) contains *SIP4* cloned in pLexA(1–202) + PL (Ruden *et al*., 1991) and was constructed in the same way as pLexA–SIP4 (Lesage *et al*., 1996).

To construct pOV30 and pOV31, we amplified by PCR the region of *SIP4* encoding the first 130 amino acids, using the template pPL40 (Lesage *et al*., 1996) and the primers 5′-GGGGGATCCCGATGGCCAA-GAGGAAATATG-3′ and 5′-GGGGGATCCTCACTGAGTGTTGTAA-TTATCAAAAC-3′ (*Bam*HI sites underlined). The resulting fragment was subcloned into the *Bam*HI site of pACTII (Li *et al*., 1994) or pGEX-3X (Pharmacia), yielding pOV30 and pOV31, respectively.

pOV29 and pOV42 contain the *SIP4* gene under the control of the *ADH1* promoter, and the *URA3* and *LEU2* markers, respectively. pOV29 and pOV42 were constructed by cloning the *Eco*RI–*Sal*I fragment from $pLexA_{202}$–SIP4 into the cognate sites of pWS93 (Song and Carlson, 1998) and pSK134 (derivative of pACTII lacking the GAD sequence; gift of S.Kuchin), respectively.

To construct pOV22, we inserted one copy of the oligonucleotide OL1 containing $CSRE_{FBP1}$ in the *Xho*I site 5′ to the promoter of the *CYC1*Δ*UAS–lacZ* fusion on plasmid pΔSS (Johnson and Herskowitz, 1985). The CSRE is in the same orientation as in the *FBP1* promoter.

β-galactosidase assays

Four different transformants were grown to mid-log phase in selective SC + 2% glucose; derepressed cultures were prepared by shifting to SC + 2% glycerol + 2% ethanol for 5 h 30 min, except where noted otherwise. β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units (Miller, 1972).

Preparation of yeast nuclear extracts

Nuclear extracts were prepared as described previously (Schneider *et al*., 1986) using cells grown to mid-log phase in selective SC + 2% glucose.

Purification of bacterially expressed proteins

$GST–Sip4_{1–130}$ and GST proteins were expressed from *E.coli* DH5α transformed with pOV31 or pGEX-3X, respectively. Protein expression was induced by the addition of 0.5 mM IPTG to the culture (10 ml) for 3 h. Bacterial pellets were resuspended in 1 ml buffer STE (10 mM

Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 100 μg of lysozyme, and incubated for 15 min on ice. After addition of 5 mM dithiothreitol and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ protease inhibitor cocktail (Boehringer Mannheim)], cells were briefly sonicated on ice. Extracts were cleared by centrifugation in a microcentrifuge for 5 min. Triton X-100 was added to 1% final concentration, and extracts were incubated with glutathione–Sepharose 4B beads (Pharmacia) at 4°C for 1 h. Beads were washed five times with 1 ml of buffer STE + 1% Triton X-100 and then once with 1 ml of buffer STE. GST or GST–$Sip4_{1–130}$ were eluted with 50 μl of 10 mM glutathione and protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad). The purified proteins were examined by SDS–polyacrylamide gel electrophoresis and Coomassie Blue staining. GST–$Sip4_{1–130}$ migrated with the expected mobility.

Electrophoretic mobility shift assay

Protein–nucleic acid complexes were allowed to form in 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, 50 mM NaCl, and 12.5% (v/v) glycerol. In a volume of 20 μl, yeast nuclear extracts (10–20 μg protein) or bacterially expressed proteins (100–200 ng) were pre-incubated with 1 μg poly(dI–dC) and 2 μg BSA for 15 min on ice. The DNA probe, labeled with the Klenow fragment of DNA polymerase I, was then added (0.05 ng, ~40 000 c.p.m.), and incubation was continued for 30 min at room temperature. Electrophoresis was performed in 4% polyacrylamide gels in 45 mM Tris-borate, 1 mM EDTA at 20 V/cm for 60 min at 4°C.

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