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CAT5, a new gene necessary for derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae*

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***PCK1* encoding phosphoenolpyruvate carboxykinase is transcriptionally regulated by two upstream activating elements. By screening for mutants that failed to derepress a $UAS2_{PCK1}$–*CYC1–lacZ* reporter gene we isolated the new recessive derepression mutation *cat5*. The *CAT5* gene encodes a protein of 272 amino acids showing a 42% identity to the ZC395.2 gene product of *Caenorhabditis elegans* whose function is unknown. Deletion of *CAT5* caused a complete loss of glucose derepression affecting gluconeogenic key enzymes. Respiration, but not mitochondrial cytochrome *c* oxidase activity, was also affected. *CAT5* expression is 5- to 6-fold repressed by glucose, and *CAT5* transcriptional activation was dependent on *CAT1* (*SNF1*), *CAT8* and *CAT5* itself. The *CAT5* gene is necessary for $UAS1_{PCK1}$ and $UAS2_{PCK1}$ protein binding since a carbon source-specific interaction was no longer detectable in *cat5* mutants. Glucose derepression of gluconeogenesis depends on the active Cat1 (Snf1) protein kinase and the Cat8 zinc cluster activator. Mig1p-independent overexpression of *CAT8* did not stimulate activation of gluconeogenic promoters in *cat1* and in *cat5* mutants. Since Cat8p multicopy expression suppresses the ethanol growth deficiency in *cat1* (*snf1*) mutants, these results indicate that activation of Cat8p by the Cat1p (Snf1p) kinase and the Cat5p protein might be necessary for release from glucose repression.**

Keywords: *CAT5*/gluconeogenesis/glucose repression/*PCK1*/*Saccharomyces cerevisiae*

Introduction

Growth of *Saccharomyces cerevisiae* on non-fermentable carbon sources (glycerol, lactate, acetate or ethanol) requires the expression of enzymes belonging to the gluconeogenic pathway and the glyoxylate cycle. The key enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK; encoded by the *PCK1* gene) and fructose-1,6-bisphosphatase (FBPase; encoded by the *FBP1* gene), are strictly regulated and are dependent on the available carbon source. Both enzymes are regulated by several biochemical and genetic regulatory systems: FBPase is allosterically inhibited by AMP and fructose-2,6-bisphosphate (Gancedo *et al.*, 1965; Lederer *et al.*, 1981) and additionally inactivated by reversible phosphorylation upon addition of glucose to the medium (Lenz and Holzer, 1980; Müller and Holzer, 1981). PEPCK is similarly phosphorylated, however, without loss of activity (Burlini *et al.*, 1989). Both enzymes are proteolytically degraded after glucose addition to cells growing with non-fermentable carbon sources (glucose inactivation) (Gancedo, 1971; Haarasilta and Oura, 1975; Gancedo and Schwerzmann, 1976) and, furthermore, transcription of both genes is subject to glucose repression (Haarasilta and Oura, 1975; Sedivy and Fraenkel, 1985).

Glucose repression is a very complex regulatory system (reviewed by Entian and Barnett, 1992; Gancedo, 1992; Johnston and Carlson, 1992; Ronne, 1995) that affects many different genes involved in the utilization of sugars different from glucose (*SUC*, *MAL* or *GAL* genes), respiratory enzymes and mitochondrial biogenesis, gluconeogenesis, the glyoxylate cycle, the citric acid cycle, β-oxidation and peroxisomal biogenesis. Proteins essential for the derepression of gluconeogenesis were identified by mutant isolation (Ciriacy, 1977; Zimmermann *et al.*, 1977; Carlson *et al.*, 1981; Entian and Zimmermann, 1982; Neigeborn and Carlson, 1984) and characterization of the respective genes (Celenza and Carlson, 1984; Schüller and Entian, 1988). Cat1p (Snf1p) is a serine/threonine protein kinase (Celenza and Carlson, 1986) and Cat3p (Snf4p) is a subunit interacting with Cat1p necessary for full catalytic activity of the protein complex (Celenza and Carlson, 1989; Fields and Song, 1989). The Cat1/Cat3 protein kinase is necessary for activation of various genes after depletion of glucose and growth on nutrients different from glucose including *SUC* gene expression, gluconeogenesis and mitochondrial gene expression (Ulery *et al.*, 1994).

The present genetic and biochemical data suggest that release from glucose repression after exhaustion of glucose in the medium (= glucose derepression) is positively regulated. Upstream activation sequence (UAS) elements were identified in the promoter regions of *FBP1* (Niederacher *et al.*, 1992; Hedges *et al.*, 1995), *ICL1* (encoding isocitrate lyase) (Schöler and Schüller, 1994), and *PCK1* (Proft *et al.*, 1995). Proteins binding in a carbon source-dependent manner to UAS elements within *FBP1* and *ICL1* promoters were characterized by gel retardation experiments (Niederacher *et al.*, 1992; Schöler and Schüller, 1994). Because of its carbon source-dependent binding activity the UAS elements were termed as CSRE for carbon source-responsive element (Schöler and Schüller, 1994). Recently a zinc cluster encoding the gene *CAT8* has been characterized as a specific derepression factor of genes encoding gluconeogenic key enzymes (Hedges *et al.*, 1995). The Cat8p is assumed to bind directly to *cis*-activators within the promoters of gluconeogenic genes. *CAT8* expression is regulated by glucose repression in two ways: (i) *CAT8* expression depends on

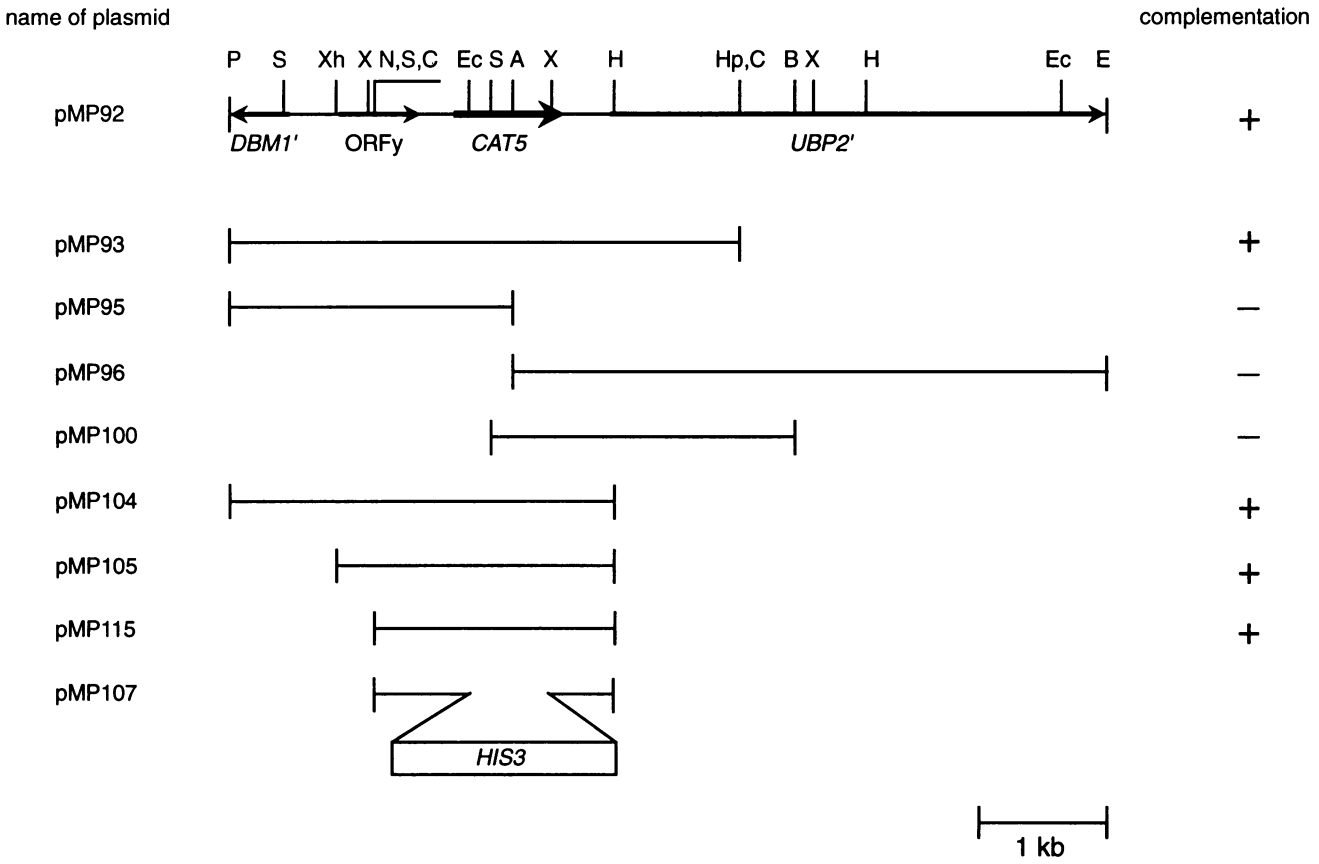

Fig. 1. Restriction map of *CAT5*. Plasmids used for complementation analysis are described in Materials and methods. Growth complementation ($YEPE_3$) is summarized on the right (+, complementation; –, no complementation). Restriction sites: A, *Asp*718; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; N, *Nsi*I; P, *Pst*I; S, *Sph*I; X, *Xba*I; Xh, *Xho*I. The *cat5*::*HIS3* disruption (pMP107) is shown in the bottom line.

the active Cat1/Cat3 (Snf1/Snf4) kinase and (ii) *CAT8* expression is repressed by the global zinc finger repressor Cat4 (Mig1) (Nehlin and Ronne, 1990; Schüller and Entian, 1991).

Intensive studies on the *PCK1* promoter revealed two carbon source-dependent CSRE motifs, $UAS1_{PCK1}$ and $UAS2_{PCK1}$, mediating strong activation of transcription on non-fermentable carbon sources (Proft *et al.*, 1995). By using the $UAS2_{PCK1}$ target sequence fused to a *CYC1–lacZ* reporter gene ($UAS2_{PCK1}$–*CYC1–lacZ*) we screened for mutants that specifically fail to activate the UAS2 element after glucose exhaustion. Beside the previously described *cat8* mutation we also found an additional mutation, *cat5*, which prevented glucose derepression of gluconeogenic enzymes. *CAT5* encodes a 31 kDa protein and is obviously essential for derepression of gluconeogenic enzymes, the glyoxylate cycle and some respiratory enzymes, as well as for the specific CSRE-responsive transcriptional activation complex.

Results

Isolation and characterization of cat5 mutants

To characterize *trans*-factors necessary for derepression of *PCK1*, wild-type cells (WAY.5-4A) transformed with the $UAS2_{PCK1}$–*CYC1–lacZ* fusion plasmid pMP53 were mutagenized and then screened for a loss of $UAS2_{PCK1}$-mediated *lacZ* gene activation (see Materials and methods). Among the 63 mutants isolated that failed to activate $UAS2_{PCK1}$, 52 were cytochrome *c* oxidase-negative and possibly defective in mitochondrial biogenesis. Only 11 mutants showed normal cytochrome *c* oxidase activity but an inability to grow with non-fermentable carbon sources. From these, 10 were allelic to the previously isolated *cat8* mutant and one mutant corresponded only to the new gene locus *cat5*. No *cat1* or *cat3* mutations were obtained by the screening. The *cat5* mutation was recessive and characterized as a single nuclear gene defect as shown by a 2:2 segregation.

Cloning of CAT5

The *cat5*-111 mutant strain (WAY.5-4A/111) was transformed with an *S.cerevisiae* genomic DNA library (YCp50-based, ATCC No 37415). Transformants were selected on glucose medium lacking uracil (SCD_2–ura), collected and then plated on medium containing ethanol ($YEPE_3$). Plasmid DNA was isolated from transformants growing on $YEPE_3$. Only one kind of plasmid carrying a genomic insert of ~16 kb was obtained which restored growth on ethanol when retransformed in the *cat5*-111 mutant. Several subclones in YCplac33 were assayed for complementation of the *cat5* growth defect (Figure 1). We sequenced the region between the already known genes *DBM1* (EMBL accession No. U07421) and *UBP2* (EMBL accession No. M94916) and identified two putative open reading frames (ORF), ORFy (238 amino acids) and *CAT5* (272 amino acids). Our complementation analysis clearly revealed the *CAT5* ORF to suppress the mutant phenotype (Figure 1). The predicted Cat5p (Figure 2) of

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   1                                                          CGATAATATTCGTCAAATGGTA   22
  23  TCTTTGATGAAGTCTTACCATATCCGTCCTATTATAATAGGACCGGGGCTAGTAGATAGAGAG   85
  86  AAGTGGGAAAAAGAAAAATCTGAAGAAATAGCTCTCGGATACTTCCGTACCAACGAGAACTTT  148
 149  GCCATTTATTCCGATGCCTTAGCAAAACTAGCCAATGAGGAAAAAGTTCCCTTCGTGGCTTTG  211
 212  AATAAGGCGTTTCAACAGGAAGGTGGTGATGCTTGGCAACAACTGCTAACAGATGGACTGCAC  274
 275  TTTTCCGGAAAAGGGTACAAAATTTTTCATGACGAATTATTGAAGGTCATTGAGACATTCTAC  337
 338  CCCCAATATCATCCCAAAAACATGCAGTACAAACTGAAAGATTGGAGAGATGTGCTAGATGAT  400
 401  GGATCTAACATAATGTCTTGAATGCAGTAATCCTTTGTACGTACTCTGTCTATATTTCCCTAA  463
 464  CTTTTAAGCAACTGTATAATTTTTCATATACGGGATTTTCAGGAAAAAAAACAATAGAAATCT  526
 527  ATAAAAC ATG TTT CCT TAT TTT TAC AGA CGA GAG TTT TAT TCT TGT GAA  575
   1          M   F   P   Y   F   Y   R   R   E   F   Y   S   C   E    14

 576  AAC GTC GTT ATC TTC TCC TCC AAA CCA ATT CAA GGA ATA AAG ATA TCA  623
  15   N   V   V   I   F   S   S   K   P   I   Q   G   I   K   I   S   30

 624  CGT ATA CGG GAG AGA TAC ATA GAA ATT ATG TTA TCC CGT GTT TCA GTT  671
  31   R   I   R   E   R   Y   I   E   I   M   L   S   R   V   S   V   46

 672  TTC AAA CCT GCC AGC AGA GGC TTT TCC GTC TTA TCA TCT TTA AAG ATA  719
  47   F   K   P   A   S   R   G   F   S   V   L   S   S   L   K   I   62

 720  ACA GAA CAT ACA TCA GCA AAA CAC ACC GAA AAA CCT GAG CAT GCT CCC  767
  63   T   E   H   T   S   A   K   H   T   E   K   P   E   H   A   P   78

 768  AAG TGT CAG AAT TTA TCA GAT GCT CAG GCT GCA TTT TTG GAC CGT GTT  815
  79   K   C   Q   N   L   S   D   A   Q   A   A   F   L   D   R   V   94

 816  ATT CGT GTA GAT CAA GCT GGC GAA TTA GGT GCA GAC TAC ATC TAC GCT  863
  95   I   R   V   D   Q   A   G   E   L   G   A   D   Y   I   Y   A  110

 864  GGC CAG TAC TTC GTG TTG GCT CAT AGG TAC CCT CAC TTG AAA CCT GTG  911
 111   G   Q   Y   F   V   L   A   H   R   Y   P   H   L   K   P   V  126

 912  CTA AAG CAC ATA TGG GAC CAG GAG ATA CAT CAT CAT AAT ACT TTT AAC  959
 127   L   K   H   I   W   D   Q   E   I   H   H   H   N   T   F   N  142

 960  AAT TTG CAA TTG AAA AGG AGA GTC AGG CCT TCC TTA TTA ACG CCT TTG 1007
 143   N   L   Q   L   K   R   R   V   R   P   S   L   L   T   P   L  158

1008  TGG AAG GCA GGA GCC TTT GCA ATG GGG GCT GGT ACC GCA TTG ATT TCT 1055
 159   W   K   A   G   A   F   A   M   G   A   G   T   A   L   I   S  174

1056  CCA GAA GCA GCT ATG GCT TGT ACT GAA GCT GTC GAG ACA GTA ATC GGA 1103
 175   P   E   A   A   M   A   C   T   E   A   V   E   T   V   I   G  190

1104  GGG CAC TAC AAT GGC CAA TTG CGA AAC TTG GCC AAT CAA TTC AAT TTA 1151
 191   G   H   Y   N   G   Q   L   R   N   L   A   N   Q   F   N   L  206

1152  GAA AGA ACA GAT GGA ACA AAG GGT CCA AGT GAG GAA ATC AAA TCC TTA 1199
 207   E   R   T   D   G   T   K   G   P   S   E   E   I   K   S   L  222

1200  ACT TCT ACT ATC CAA CAG TTC AGG GAT GAC GAG CTA GAG CAT CTA GAC 1247
 223   T   S   T   I   Q   Q   F   R   D   D   E   L   E   H   L   D  238

1248  ACC GCT ATC AAG CAT GAT TCG TAT ATG GCA GTT CCA TAT ACA GTT ATC 1295
 239   T   A   I   K   H   D   S   Y   M   A   V   P   Y   T   V   I  254

1296  ACT GAA GGT ATT AAA ACG ATT TGC AGA GTA GCT ATA TGG AGT GCC GAA 1343
 255   T   E   G   I   K   T   I   C   R   V   A   I   W   S   A   E  270

1344  AGA ATT TAA CCACCAGAAAGTGGCATACATCAGTCGCGTTATGCCAGAAAAGGAGAATTG 1403
 271   R   I   *
1404  AAAGGAAAACGGTTTGATAAATGTCCTAATTAAACTATCATGTATAAAATTATGTATCATCCT 1466
1467  TACGCATTTTAACGCTATATGACCAATATGACAGGAATAGATACACTGTCTATAATTATGTAA 1529
1530  ATGGGGTATGGGTTCATAGTCTAAGGGTGAGTACAAACTGGATCTTTAACAAGAGTAACAGTT 1592
1593  AATTAGAGCAAAACTATAGTACATATAGCTTGAAAAAAA                          1631
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Fig. 2. Nucleotide sequence of *CAT5* and deduced amino acid sequence.

30 920 Da shows homologies to the *Caenorhabditis elegans* ZC395.2 gene product which was identified through the *Caenorhabditis* genome project (EMBL accession No. U13642). The deduced *C.elegans* amino acid sequence has a 42% identity over 187 amino acids with the yeast *CAT5* gene. Furthermore both proteins were similar in a hydrophobicity analysis indicating that they may be functionally related (Figure 3). The Cat5p has a predicted isoelectric point of 8.78, and the codon adaption index (CAI) is rather low at 0.17. *CAT5* is located on

A

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CAT5      86 AQAAFLDRVIRVDQAGELGADYIYAGQYFVLAHRYPHLKPVLKHIWDQEIHHHNTFNNLQLKRRVRPSLLTPLW 159
             ++ A ++++IRVD AGELGAD IYAGQ  VL         V+K +WD+E  H +T   L  K  V  ++ +P++
ZC395.2   14 SRQALIEKIIRVDHAGELGADRIYAGQLAVLQGSSVGS--VIKKMWDEEKEHLDTMERLAAKHNVPHTVFSPVF  85

CAT5     160 KAGAFAMGAGTALISPEAAMACTEAVETVIGGHYNGQLRNLANQFNLERTDGTKGPSEEIKSLTSTIQQFRDDE 233
                A+A+G G+AL+  E AMACT AVE +IG HYN QL+ L               E  K L   + + RD+E
ZC395.2   86 SVAAYALGVGSALLGKEGAMACTIAVEELIGQHYNDQLKELLADDP----------ETHKELLKILTRLRDEE 148

CAT5     234 LEHLDTAIKHDSYMAVPYTVITEGIKTICRVAIWSAERI 272
             L H DT ++HD   A  Y+ +   I+T C+ AI  AE+I
ZC395.2  149 LHHHDTGVEHDGMKAPAYSALKWIIQTGCKGAIAIAEKI 187
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B

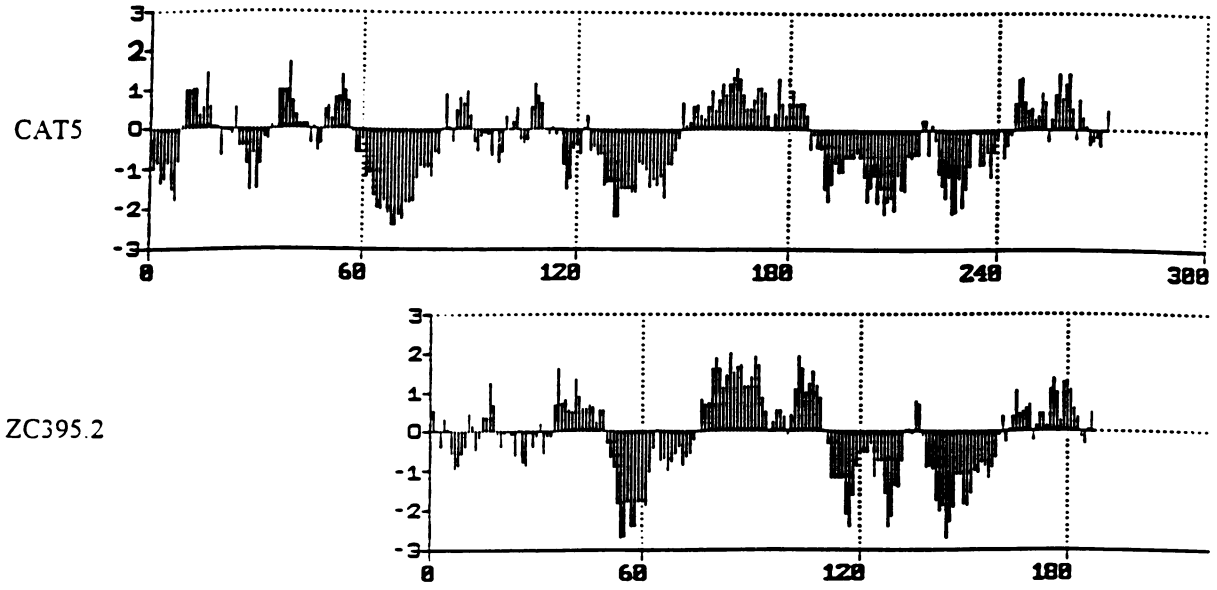

Fig. 3. Comparison of Cat5p with the ZC395.2 gene product of *C.elegans*. (**A**) Amino acid similarities: identical amino acids are given with the respective letters, similar amino acids are marked with +. (**B**) Hydrophobicity analysis: positive values are hydrophobic groups, negative values are hydrophilic groups.

chromosome XV; mapping experiments placed *DBM1*, which is adjacent to *CAT5*, on chromosome XV.

Influence of a Δcat5 mutation on glucose repression/derepression of PCK1

We replaced most of the Cat5p encoding region (~76%) by substituting it with *HIS3* (Figure 1) to determine phenotypes caused by the loss of *CAT5* gene function. The resulting *cat5::HIS3* mutants did not grow on non-fermentable carbon sources. We then crossed the *cat5::HIS3* strain with the original *cat5* mutant; however, the resultant diploids were unable to grow on ethanol as a carbon source and failed to sporulate. Deletion of *CAT5* resulted in a loss of O_2 consumption, measured with whole cells, therefore indicating that Cat5p is globally involved in respiration whereas functional cytochrome *c* oxidase was still detectable similar to the wild-type. By using episomal *PCK1–lacZ* fusion plasmid pPEPCKlacZ (Proft *et al.*, 1995) and heterologous test constructs $UAS1_{PCK1}$–*CYC1–lacZ* (pMP57) and $UAS2_{PCK1}$–*CYC1–lacZ* (pMP53) (Proft *et al.*, 1995), the new *cat5* deletion mutant and other mutants affected in the glucose repression/derepression system were examined for their ability to activate *PCK1* gene expression (for plasmid constructions see Materials and methods). As expected the glucose derepression genes *CAT1* (*SNF1*, *CCR1*), *CAT3* (*SNF4*) and the recently identified *CAT8* gene were essential for activation of the *PCK1*–β-galactosidase gene fusion (Tables I and II). The newly identified *cat5* mutants also failed to activate the *PCK1–lacZ* reporter plasmid and were also unable to derepress all gluconeogenic enzymes tested whereas glucose-dependent regulation of invertase and maltase was normal (Table III). Cat4 (Mig1) is a zinc finger containing repressor known to be involved in regulation of various glucose-repressible genes (*GAL*, *MAL*, *SUC*, *CAT8*) by direct binding to the respective upstream regions (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991; Schüller and Entian, 1991; Hedges *et al.*, 1995).

Elevated expression levels in *cat4* (*mig1*) deletion mutants while growing with glucose could not be observed using *PCK1–lacZ*. Mutations in other genes that exert glucose repression also had no or only slight effects on the regulated expression patterns of the *PCK1*–β-galactosidase construct. We tested the repression genes *CAT80* (*GRR1*, encoding a leucine-rich protein; Entian and Zimmermann, 1980; Flick and Johnston, 1991), *HEX2* (*REG1*, encoding a nuclear negative effector; Niederacher and Entian, 1991), *HXK2* (encoding hexokinase isoenzyme PII; Entian, 1980; Fröhlich *et al.*, 1984) and *TUP1* (*FLK1*, *UMR7*, *CYC9*, *AMM1*, *SLF2*, *AER1*)/*CYC8* (*SSN6*) (encoding a nuclear repressor complex with pleiotropic effects; Williams *et al.*, 1991; Keleher *et al.*, 1992). Similar results have been obtained for UAS_{PCK1}-mediated gene activation with the respective *cis*-activators from *PCK1* inserted into a heterologous *CYC1–lacZ* reporter gene (pMP53, pMP57, Table II). The measurements clearly characterized glucose derepression mutants *cat1*, *cat3*, *cat5* and *cat8* acting through $UAS1_{PCK1}$ and $UAS2_{PCK1}$. No comparable *trans* influence was observed for any of the other repression mutants tested. Derepression was less efficient in mutants *hex2*, *reg1* and *hxk2*.

Table I. Influence of regulatory mutations on expression of a *PCK1–lacZ* reporter gene

Strain[a]	Relevant genotype	Specific activity β-galactosidase (nmol/min/mg) with	
		Glucose[b]	Ethanol[c]
WAY.5-4A	Wild-type	54	9462
JS87.11-17C	*cat1 (snf1)*	3	5
JS89.1-1	*cat3 (snf4)*	10	17
CEN.MP3-1A	*cat5*	5	6
ENY.DG1-2B	*cat8*	3	52
WAY.PK18-1	*cat4 (mig1)*	22	7288
WAY.JF1	*cat80*	224	8737
ENY.hex2-3A	*hex2*	3	2784
WAY.6-2B	*hxk2*	68	1138
ENY.tup1-7A	*tup1*	36	8099
ENY.DG5-1C	*cyc8*	318	16 362

[a]The strains indicated were transformed with the episomal reporter plasmid pPEPCKlacZ (*PCK1–lacZ URA3* 2μm).
[b]Repressed growth conditions (SCD_4 medium lacking uracil).
[c]Derepressed growth conditions (SCE_3 medium lacking uracil).

Role of CAT5 in carbon source-responsive transcriptional activation of PCK1

We tested the effects of a Δ*cat5* deletion mutation on the CSRE-dependent formation of protein–DNA complexes with synthetic oligonucleotides representing $UAS1_{PCK1}$ and $UAS2_{PCK1}$. As shown in Figure 4 three different protein–DNA complexes (CI, CII and CIII) were observed with protein extracts from wild-type cells using oligonucleotides OMP31/32 (= $UAS1_{PCK1}$) and OMP33/34 (= $UAS2_{PCK1}$). Specificity of these interactions was shown by competition with an excess of unlabelled OMP31/32

Table II. $UAS1_{PCK1}$ ($UAS2_{PCK1}$)-mediated gene activation in regulatory mutants

Strain	Relevant genotype	Specific activity β-galactosidase (nmol/min/mg) with			
		Glucose[b]		Ethanol[c]	
		$UAS1_{PCK1}$[d]	$UAS2_{PCK1}$[e]	$UAS1_{PCK1}$	$UAS2_{PCK1}$
WAY.5-4A	Wild-type	31	43	3248	4525
JS87.11-17C	*cat1 (snf1)*	6	14	16	32
JS89.1-1	*cat3 (snf4)*	40	78	88	304
CEN.MP3-1A	*cat5*	13	23	16	24
ENY.DGY.1-2B	*cat8*	8	9	45	25
WAY.PK18-1	*cat4 (mig1)*	19	48	1042	1822
WAY.JF1	*cat80*	147	165	4774	5179
ENY.hex2-3A	*hex2*	7	8	876	2623
WAY.6-2B	*hxk2*	12	49	904	2749
ENY.tup1-7A	*tup1*	89	216	2639	3092
ENY.DG5-1C	*cyc8*	62	109	1461	1945

[a]The strains indicated were transformed with the respective UAS_{PCK1}–*CYC1–lacZ* fusion plasmids (*URA3* 2μm).
[b]Repressed growth conditions (SCD_4 medium lacking uracil).
[c]Derepressed growth conditions (SCE_3 medium lacking uracil).
[d]$UAS1_{PCK1}$–*CYC1–lacZ* fusion pMP57 was used.
[e]$UAS2_{PCK1}$–*CYC1–lacZ* fusion pMP53 was used.

Table III. Enzyme activities of the wild-type and a *cat5* deletion mutant

Strain	Relevant genotype	Specific activity (nmol of substrate/min/mg)									
		PEPCK		ICL		FBPase		Invertase		Maltase	
		D	E	D	E	D	E	D	R	D	M
MC.999-1A	Wild-type	7	379	1	105	3	34	18	698	17	1308
MPY.3	*cat5::HIS3*	5	20	1	6	3	3	8	1015	11	2098

Enzyme activities were determined in repressed cells (D = growth on yeast extract–peptone–dextrose, YEPD) and in derepressed cells (E = growth on yeast extract–peptone–ethanol, YEPE; R = growth on yeast extract–peptone–raffinose, YEPR; M = growth on yeast extract–peptone–maltose, YEPM).

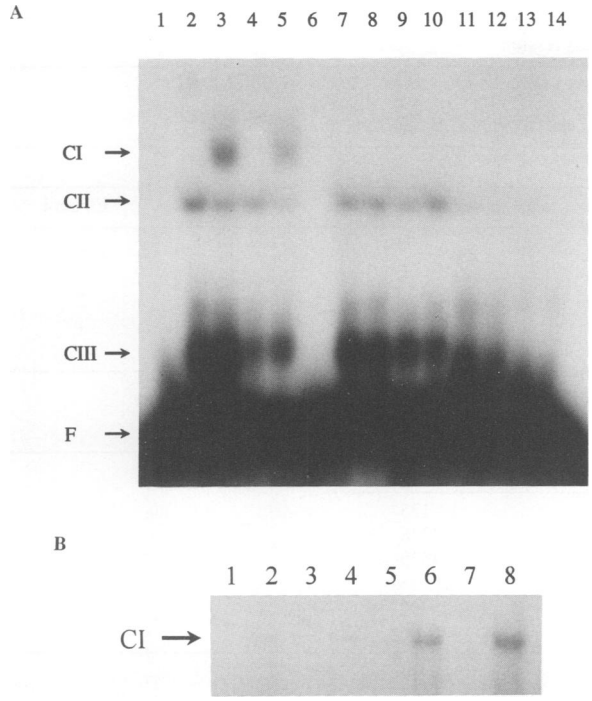

Fig. 4. Specific protein binding on UAS_{PCK1} elements. (**A**) Gel retardation experiments with UAS_{PCK1} probes OMP31/32 (lanes 1, 2, 3, 7 and 8) and OMP33/34 (lanes 4, 5, 6, 9 and 10). Each probe contained 20 000 c.p.m. of ^{32}P-labelled oligonuleotide. Binding reactions were performed with 30 μg of total cellular protein. A 100-fold molar excess of unlabelled oligonucleotide was used for competition. Lanes 1 and 6, no protein added; lanes 2 and 4, protein extract from repressed wild-type cells; lanes 3 and 5, protein extract from derepressed wild-type cells; lanes 7 and 9, protein extracts from repressed Δ*cat5* mutant cells; lanes 8 and 10, protein extract from derepressed Δ*cat5* mutant cells; lanes 11–14, like lanes 2–5 competition with OMP31/32 and OMP33/34, respectively. CI, CII and CIII, protein–DNA complexes; F, free DNA. (**B**) Gel retardation experiments with $UAS1_{PCK1}$ probe OMP31/32. Conditions were the same as in A. Lanes 1 and 2, protein extracts from repressed and derepressed Δ*cat5* cells, respectively; lanes 3 and 4, repressed and derepressed Δ*cat5* cells with multicopy *CAT8* (pMP121), respectively; lanes 5 and 6, repressed and derepressed wild-type, respectively; lanes 7 and 8, like lanes 5 and 6, competition with unspecific OMP1/2.

(OMP33/34) (Figure 4A, lanes 11–14). Interestingly, CII and CIII were present with protein extracts from glucose- as well as from ethanol-grown cells, whereas CI exclusively occurred with protein extracts from derepressed cells. This result strongly indicates that protein(s) involved in forming CI have a specific function for the UAS_{PCK1}-mediated derepression of gluconeogenic *PCK1* similar to those previously described for *FBP1* and *ICL1*. In Δ*cat5* mutants, however, the CI protein–DNA complex was absent (Figure 4A, lanes 8 and 10) whereas protein–DNA complexes CII and CIII were not affected. After transformation of the Δ*cat5* mutants with plasmid-encoded *CAT5* gene all three protein–DNA complexes were present again, however, the signal representing CI was not stronger after multicopy expression of *CAT5* (data not shown). These results suggested that *CAT5* may not directly bind to the UAS_{PCK1} elements and only indirectly mediates the formation of the CSRE-dependent transcriptional activation complex. This result is in accordance with the failure to detect a transcriptional activation activity of Cat5p after its fusion to the Gal4p DNA binding domain (see Materials and methods, data not shown). Furthermore we could not restore CI complex formation with overexpression of *CAT8* (Figure 4B, lanes 3 and 4). We also proved the sequence specificity of the UAS_{PCK1} protein complex CI by using an excess of unlabelled oligonucleotide with unspecific sequence (Figure 4B, lanes 7 and 8).

Table IV. Influence of regulatory mutations on expression of a *CAT5–lacZ* reporter gene

Strain[a]	Relevant genotype	Specific activity β-galactosidase (nmol/min/mg) with	
		Glucose[b]	Ethanol[c]
WAY.5-4A	Wild-type	43	240
JS87.11-17C	*cat1 (snf1)*	7	8
CEN.MP3-1A	*cat5*	11	16
ENY.DG1-2B	*cat8*	10	21
WAY.PK18-1	*cat4 (mig1)*	78	448

[a]The strains indicated were transformed with the episomal reporter plasmid pMP 113 (*CAT5–lacZ URA3* 2μm).
[b]Repressed growth conditions (SCD_4 medium lacking uracil).
[c]Derepressed growth conditions (SCE_3 medium lacking uracil).

Transcriptional regulation of CAT5

To study the expression of *CAT5* we constructed an episomal *CAT5–lacZ* fusion plasmid. Plasmid pMP113 contained 580 bp of the *CAT5* upstream region and the first 235 codons of the *CAT5* gene fused in-frame to the β-galactosidase gene. As shown in Table IV the *CAT5* promoter fused to *lacZ* gave only moderately expressed β-galactosidase activities but significantly responded to the carbon source and was 5- to 6-fold more active under derepressed growth conditions. Furthermore we tested glucose repression/derepression mutants *cat1* (*snf1*), *cat5*, *cat8* and *cat4* (*mig1*) for *CAT5–lacZ* expression. *CAT1* (*SNF1*), *CAT5* and *CAT8* gene function was required to derepress *CAT5* while the loss of *CAT4* (*MIG1*) had no effect on the regulation of a *CAT5–lacZ* reporter gene.

Influence of cat5 mutation on CAT8 transcriptional regulation

Since the zinc cluster containing *CAT8* is assumed to be the central transcriptional activator specific for gluconeogenic derepression, we assayed the effect of a *cat5* deletion on an episomal *CAT8–lacZ* reporter plasmid (pDG212, see Materials and methods). The *CAT8*–β-galactosidase fusion was derepressed 150-fold after growth with ethanol in wild-type cells (12 nmol/min/mg on glucose and 1801 nmol/min/mg on ethanol). However, in Δ*cat5* mutants *CAT8–lacZ* derepression was strongly reduced and 9-fold decreased as compared with the wild-type (6 nmol/min/mg on glucose and 96 nmol/min/mg on ethanol). These data suggest that *CAT5*, like *CAT1* (*SNF1*) and *CAT3* (*SNF4*) (Hedges *et al.*, 1995), is necessary for full derepression of *CAT8* transcription.

Influence of CAT8 overexpression on gluconeogenic regulation

CAT8 encodes a zinc cluster protein necessary for the activation of gluconeogenic genes (Hedges *et al.*, 1995). *CAT8* itself is strongly regulated by glucose repression, intact Cat1/Cat3 (Snf1/Snf4) protein kinase is absolutely

Table V. Influence of constitutive *CAT8* overexpression on gluconeogenic gene expression

A

Strain/multicopy *CAT8*[a]	Relevant genotype	Specific activity (nmol of substrate/min/mg)			
		PEPCK		ICL	
		Glucose	Ethanol	Glucose	Ethanol
CEN.PK2-1A	wild-type	2	390	2	125
ENY.DG1-2B	*cat8*	4	264	3	83
CEN.MP3-1A	*cat5*	3	12	2	2
JS87.11-17C	*cat1 (snf1)*	3	13	2	4

B

Multicopy *CAT8*[d]	Reporter construct	Specific activity β-galactosidase (nmol/min/mg) with	
		Glucose[b]	Ethanol[c]
–	*PCK–lacZ*	55	13 125
–	$UAS1_{PCK1}$–*CYC1–lacZ*	31	3248
–	$UAS2_{PCK1}$–*CYC1–lacZ*	43	4525
+	*PCK1–lacZ*	76	12 258
+	$UAS1_{PCK1}$–*CYC1–lacZ*	152	5040
+	$UAS2_{PCK1}$–*CYC1–lacZ*	389	5169

[a]Episomal *ACT1–CAT8* fusion plasmid was used (pMP121).
[b]Repressed growth on synthetic complete glucose medium lacking uracil or uracil/leucine.
[c]Derepressed growth on synthetic complete ethanol medium lacking uracil or uracil/leucine.
[d]Wild-type strain CEN.PK2-1A was used with or without pMP121.

necessary for *CAT8* transcriptional activation. To characterize the roles of Cat1p and Cat8p in gluconeogenic regulation we constitutively overexpressed *CAT8* under the control of the *ACT1* promoter (encoding actin) using an episomal vector (YEpACT1, see Materials and methods). The *ACT1–CAT8* fusion plasmid (pMP121, see Materials and methods) was tested for stable Cat8p overexpression by Western blot analysis. The *ACT1–CAT8* fusion construct led to a high Cat8p expression in comparable amounts in wild-type, as well as in Δ*cat5* mutants, independently on the carbon source (shown by the presence of Cat8p-specific antibodies, data not shown) and restored growth with ethanol in a Δ*cat8* deletion mutant. The inability to grow on ethanol of Δ*cat1* mutants, but not of Δ*cat5* mutants, was suppressed by constitutive *CAT8* overexpression (data not shown). As shown in Table V(A), specific activities of PEPCK and ICL were restored in a Δ*cat8* strain transformed with pMP121 indicating a functional *CAT8* expression. However, in Δ*cat1* and Δ*cat5* cells the *CAT8* overexpression could not suppress the inability to derepress gluconeogenic enzymes PEPCK and ICL. These results show that Cat5p like Cat1p (Snf1p) does not act via *CAT8* expression alone although *CAT8* expression is strongly reduced in Δ*cat5* and Δ*cat1* mutants. Moreover we co-transformed wild-type cells with *PCK1–lacZ* and the respective UAS_{PCK1}–*CYC1–lacZ* reporter plasmids (together with pMP121) to study the multicopy effect of Cat8 on the gluconeogenic *PCK1* promoter. As shown in Table V(B), constitutive *CAT8* overexpression did not affect *PCK1–lacZ* gene expression and only a slight increase of β-galactosidase levels was detectable in repressed cells using the UAS_{PCK1}–*CYC1–lacZ* reporter genes. Partial derepression on high glucose using UAS_{PCK1} elements with overexpressed *CAT8* might be due to the absence of the negative regulatory element URS_{PCK1} (Proft *et al.*, 1995) in these reporter genes. Nevertheless the *PCK1* promoter remained fully glucose repressible which strongly suggests that derepression of gluconeogenesis additionally to expression of *CAT8* needs further activation of Cat8p and/or further derepression factors dependent on active Cat1p/Cat3p and Cat5p

Table VI. Comparison of functionally tested UAS sequences within gluconeogenic promoters

Name of element	Sequence	Position
$UAS1_{PCK1}$	CGGGTGAATGGA	–562/–551
$UAS2_{PCK1}$	CGGATGAAAGGA	–471/–482
$CSRE_{ICL1}$	CGGATGAATGGA	–399/–388
$UAS2_{FBP1}$	CGGACGGATGGA	–505/–494
$UAS1_{FBP1}$	CGGACACCCGGA	–432/–421
Consensus	CGGRYRNMNGGA	

Discussion

The present investigation describes the isolation of a new regulatory element specifically involved in the derepression of gluconeogenic and glyoxylate cycle enzymes. The *CAT5* gene was identified in a screen for mutants that fail to activate the CSRE element $UAS2_{PCK1}$ in a heterologous reporter fusion. The biochemical analysis of the *cat5* mutant allele as well as that of *cat5* deletion mutants (*Δcat5*) revealed that these mutants could not derepress gluconeogenic genes *FBP1*, *PCK1* as well as the glyoxylate cycle gene *ICL1*. The *cat5* mutant also failed to derepress all CSRE elements of gluconeogenic promoters tested such as $UAS1_{PCK1}$, $UAS2_{PCK2}$, $UAS1_{FBP1}$ and $UAS2_{FBP1}$ which, in addition to their sequence similarities (see Table VI), suggests that all these elements are regulated in common.

Further analysis showed that the carbon source-specific

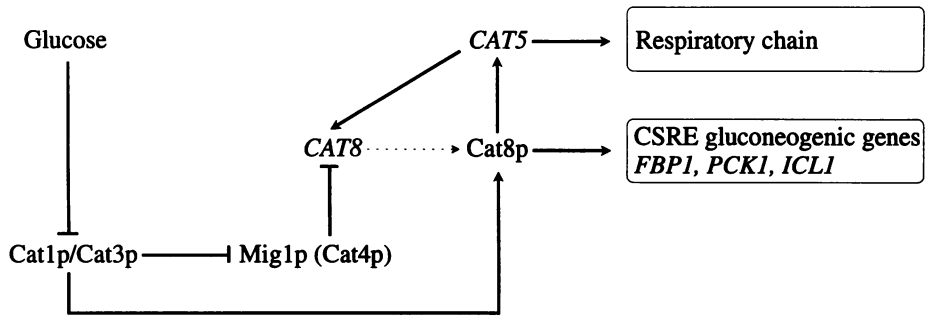

Fig. 5. Model for glucose repression/derepression.

binding to $UAS1_{PCK1}$ and $UAS2_{PCK1}$ is missing in *cat5* mutants. Whereas the carbon source-specific protein–DNA complex CI did not occur in *cat5* mutants, the carbon source-independent protein–DNA complexes CII and CIII were present as in the wild-type. The control experiments by using unlabelled UAS_{PCK1} oligonucleotides clearly showed the specificity of these three protein–DNA complexes. This observation suggests that *CAT5* encodes either a protein that is directly involved in the formation of the CSRE–protein complex or a protein that indirectly affects the formation of the respective complex. The intensity of the derepression complex did not increase if *CAT5* was expressed on multicopy plasmids and a *CAT5* fusion construct with the GAL4 DNA binding domain failed to show a gene activating function of Cat5p. These results, as well as the absence of any amino acid motifs that indicated a gene activating function, suggest that *CAT5* has an indirect influence on derepression specific gene activation. It is unlikely, but cannot be excluded, that either other proteins are rate limiting or that Cat5p is part of the CI complex without a characteristic motif for DNA binding or transcriptional activation.

Expression of the recently identified *CAT8* gene, which is a zinc cluster protein and may encode the carbon source-specific CSRE binding protein, was decreased by 9-fold in *cat5* mutants. This indicated that the failure to form CSRE complex CI in *cat5* mutants resulted from strongly reduced expression of the transcriptional activator. This, however, was disproven by using an *ACT1–CAT8* fusion which made *CAT8* expression carbon source-independent. Expression of the *ACT1–CAT8* fusion gene in a *cat5* mutant did not suppress the growth deficiency and the formation of the CSRE-specific complex CI and gluconeogenic enzymes still were derepression negative. Obviously, *CAT5* acts on *CAT8* and the gluconeogenic promoters as well.

Beside its *CAT5*-dependent activation, *CAT8* expression is also negatively regulated by the Mig1p (= Cat4p) protein and needs an active Cat1p/Cat3p (= Snf1p/Snf4p) protein kinase (Hedges *et al.*, 1995). To characterize further this genetic interaction the carbon source-independent *ACT1–CAT8* construct was also investigated in *cat1* and *cat8* mutants. In both mutants the constitutive overexpression of *CAT8* restored growth on ethanol although the gluconeogenic enzymes were not derepressed to a detectable activity in *cat1* mutants. By using the same construct in the wild-type together with $UAS1_{PCK1}$– and $UAS2_{PCK1}$–*lacZ*, respectively, we still observed nearly fully glucose repressed activities.

Overexpression of transcriptional activator *CAT8* suppressed the growth phenotype of *cat1* (*snf1*) mutants which indicated that both proteins belong to the same regulatory cascade possibly with Cat8p being a substrate of the Cat1p/Cat3p (Snf1p/Snf4p) protein kinase. The fact that although *cat1* (*snf1*) mutants grow on ethanol with high levels of Cat8p and no measurable increase in gluconeogenic enzyme activity occurred, indicates a possible role of Cat8p in the regulation of other co-regulated enzymes necessary for gluconeogenic growth.

Our present model suggests that *CAT5* represents a new regulatory element which is necessary for transcriptional activation of the *CAT8* gene, gluconeogenic and glyoxylate cycle genes. An exciting new finding is that *CAT5* expression strongly depends on *CAT1* (*SNF1*), *CAT8* and *CAT5* genes which suggests that *CAT5*, in addition to the gluconeogenic genes, is also a target of the *CAT1/CAT3* (*SNF1/SNF4*)–*CAT8* regulation pathway.

Recently *CAT5* was independently isolated by complementing a *coq7* mutant (Tzagoloff and Dieckmann, 1990) deficient in coenzyme Q (ubiquinone) biosynthesis (C.F.Clarke, personal communication). The expression of the *S.cerevisiae* respiratory chain and especially the coenzyme Q biosynthesis are glucose-repressible (Sippel *et al.*, 1983), as well as the global regulation of mitochondrial transcription being dependent on repression/derepression growth conditions (Ulery *et al.*, 1994). Therefore a co-regulation of respiratory chain elements, the biogenesis of mitochondria at all and gluconeogenesis using common regulatory elements seems conceivable. Interestingly some *cat8* mutant alleles showed a petite phenotype, resulting in a loss of cytochrome *c* oxidase and O_2 uptake activity (D.Hedges, personal communication), suggesting that Cat8p activation may globally affect respiratory metabolism. This makes *CAT5* a likely candidate which connects glucose derepression and respiration. Possibly, the Cat1p/Cat3p (Snf1p/Snf4p) protein kinase and the Cat8p transcriptional activator regulate derepression of ubiquinone via Cat5p, and Cat5p senses back the respiratory state of the cell to the derepressing machinery (see Figure 5).

The 31 kDa Cat5 protein showed significant homologies to the *C.elegans* protein ZC395.2 identified within the *C.elegans* sequencing project. Other yeast proteins such as Cat1p (Snf1p), Cat3p (Snf4p) and Sip1p, which are involved in glucose derepression, also revealed homologous counterparts in higher eukaryotes. The Cat1p (Snf1p) homologous gene *RKIN1* was isolated from rye endosperm cDNA and could even complement the *cat1* (*snf1*) mutation (Alderson *et al.*, 1991). The AMPK protein

Table VII. *Saccharomyces cerevisiae* strains used

Strain	Genotype[a]
WAY.5-4A	*MATa ura3-52 his3-Δ1 MAL2-8^c MAL3 SUC3*
MC.999-1A	*MATa ura3-52 his3-Δ1 leu2-3,112 trp1 MAL2-8^c MAL3 SUC3*
CEN.PK2-1A	*MATa ura3-52 his3-Δ1 leu2-3,112 trp1 MAL2-8^c MAL3 SUC3*
JS87.11-17C	*MATα ura3-52 leu2-3,112 cat1::HIS3 MAL2-8^c MAL3 SUC3*
JS89. 1-1	*MATα ura3-52 cat3::LEU2 MAL2-8^c MAL3 SUC3*
CEN.MP3-1A	*MATa ura3-52 leu2-3,112 trp1 cat5::HIS3 MAL2-8^c MAL3 SUC3*
ENY.DG1-2B	*MATα ura3-52 leu2-3,112cat8::HIS3 MAL2-8^c MAL3 SUC3*
WAY.PK18-1	*MATa ura3-52cat4::HIS3 MAL2-8^c MAL3 SUC3*
WAY.JF1	*MATa ura3-52 cat80::HIS3 MAL2-8^c MAL3 SUC3*
ENY.hex2-3A	*MATa ura3-52 leu2-3,112hex2-3 MAL2-8^c MAL3 SUC3*
WAY.6-2B	*MATα ura3-52hxk2::LEU2 MAL2-8^c MAL3 SUC3*
ENY.tup1-7A	*MATα ura3-52 his3-Δ1 leu2-3,112trp1 tup1-100 MAL2-8^c MAL3 SUC3*
ENY.DG5-1C	*MATα ura3-52 his3-Δ1 cyc8-20MAL2-8^c MAL3 SUC3*
WAY.5-4A/111	*MATa ura3-52 his3-Δ1cat5-111 MAL2-8^c MAL3 SUC3*

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

kinase found in liver cells is also significantly homologous to Cat1p (Snf1p) (Carling *et al.*, 1994) and more than that, AMPK was additionally associated with proteins sharing homologies with Cat3p (Snf4p) and Sip1p (Stapleton *et al.*, 1994). In mammalian cells the AMPK protein kinase regulates fatty acid metabolism which is of central importance in liver cells during carbon source starvation; a situation similar to that in yeast growing with ethanol. The further investigation of *CAT5* function in derepression of gluconeogenic enzymes will hopefully also unravel the ZC395.2 nematode function.

Materials and methods

Strains and media

The *S.cerevisiae* strains used are listed in Table VII. For amplification of recombinant plasmids *Escherichia coli* DH5α [F$^-$ (Φ *80d/lacZΔ M15*) *Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*] was used.

The composition of yeast rich medium (yeast extract and peptone-based) and synthetic complete (SC) medium have been previously described (Niederacher and Entian, 1987). As a carbon source 4% glucose, 4% raffinose, 4% maltose or 3% ethanol was added. For derepression cells were grown in glucose medium to mid-log phase and then transferred to medium with the respective non-fermentable carbon source for 6–8 h.

For screening of mutants, derepression conditions were achieved by growth in SC medium with 3% ethanol and 0.2% glucose ($SCD_{0.2}E_3$). Detection of β-galactosidase on plates was performed by addition of 80 μg of X-Gal [5-bromo-4-chloro-3-indolyl-(-D-galactoside)] per ml SC medium, which was buffered to pH 7 with 0.1 M MES.

Escherichia coli strains were grown in LB broth (Gibco). For selection of antibiotic resistant transformants, ampicillin (40 μg/ml) was added.

Plasmid constructions

PCK1–lacZ fusion plasmid pPEPCKlacZ and heterologous UAS_{PCK1}–*CYC1–lacZ* test constructs pMP53 and pMP57 are described by Proft *et al.* (1995). By complementing the *cat5*-111 mutation an *S.cerevisiae* genomic insert of ~16 kb in YCp50 was obtained containing the entire *CAT5* gene. Plasmids pMP92, pMP93, pMP95, pMP96, pMP100, pMP104, pMP105 and pMP115 were constructed by subcloning parts of this genomic insert into YCplac33 (Gietz and Sugino, 1988). The cloning strategy is shown in Figure 1.

CAT5–lacZ fusion pMP113 was obtained by ligation of the *CAT5 Nsi*I–*Xba*I fragment (pMP105) with *lacZ* fusion plasmid YEp357R (Myers *et al.*, 1986). The plasmid contains 580 bp of the *CAT5* promoter fused to *lacZ* (see Table IV). *CAT8–lacZ* fusion pDG212 was constructed by ligation of a *CAT8 Hin*dIII fragment with YEp356 (Myers *et al.*, 1986). Plasmid pDG212 contains ~1.8 kb of the *CAT8* upstream region fused with the first 66 codons of the *CAT8* gene to *lacZ*.

For constitutive *CAT8* overexpression the episomal construct YEpA-CT1 (kindly provided by F.Randez-Gil) was used containing the *ACT1* promoter inserted in YEplac181 (Gietz and Sugino, 1988). The whole *CAT8* ORF was amplified by PCR and then inserted into YEpACT1 resulting in plasmid pMP121.

To test a transcriptional activating ability of *CAT5* the entire *CAT5* gene was amplified by PCR and then fused in-frame to the *GAL4* DNA binding domain (GAL4bd) by inserting in pGBT9 (Clontech, USA). This construct restored the growth deficiency in *Δcat5* deletion strain CEN.MP3-TA and therefore was thought to be functional. Transcriptional activation of this *CAT5–GAL4*bd fusion was assayed by measuring β-galactosidase activities after transformation into test strain SFY.526 (Clontech, USA).

Mutagenesis and isolation of mutants

Yeast wild-type strain WAY.5-4A, transformed with pMP53, was grown in SCD–uracil medium to the mid-log phase. For isolation of $UAS2_{PCK1}$ derepression mutants, cells were mutagenized with 2% ethyl methanesulfonate in water for 50 min (survival rate ~10%) and washed twice with sterile water. After 6 h of incubation in SCD–uracil medium, cells were plated on $SCD_{0.2}E_3$–uracil plates containing X-Gal. White colonies were collected and rescreened. After loss of the test construct (pMP53) putative mutants were retransformed with pMP53 to exclude mutagenic effects on the used plasmid. The mutants were tested for β-galactosidase activity. Those mutants affected in their β-galactosidase activity under derepression conditions, but with intact mitochondrial cytochrome *c* oxidase, were further investigated. To show cytochrome *c* oxidase activity, cells were lysed on Whatman 3MM filters with 0.5% Tween 20 in 0.04 M potassium phosphate buffer pH 6.7 and then incubated with 1% TMPD (tetramethyl-*p*-phenylendiamine).

Construction of a cat5::HIS3 null allele

A 1.75 kb *Hin*dIII–*Nsi*I fragment carrying the *CAT5* gene was subcloned into pBluescriptIISK-Δ*Xba*I (Stratagene, modified), yielding pMP106. A 1.8 kb *Eco*RV–*Xba*I fragment containing *HIS3* was inserted into *Eco*RV–*Xba*I-cleaved pMP106 to obtain pMP107. Yeast wild-type strain MC.999-1A was integratively transformed with pMP107. Disruption of the chromosomal *CAT5* locus was verified by PCR and Southern blot analysis.

DNA sequencing

Restriction fragments were cloned in vector pBluescriptIISK$^-$, and the sequence was determined by the method of Sanger *et al.* (1977) with reverse and M13–20 primers by using DNA sequencer 373 (Applied Biosystems). The sequence of other parts of the *CAT5* gene was determined by synthetic oligonucleotide primed sequencing.

Recombinant DNA procedures

For standard recombinant DNA techniques, established protocols were followed (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). For routinely

performed PCR, *Taq* polymerase from Gibco was used. Yeast transformation was performed as described by Klebe *et al.* (1983). Isolation of plasmid DNA from yeast cells was performed as described by Hoffman and Winston (1987).

Enzyme assays

Crude extracts were prepared with glass beads (Ciriacy, 1975) and protein was determined by the microbiuret method (Zamenhoff, 1957) at 290 nm with bovine serum albumin as a standard. Invertase and maltase were assayed as previously described (Niederacher and Entian, 1987). β-Galactosidase was measured as described by Guarente (1983). Specific β-galactosidase activities given in the tables represent mean values from at least four independent measurements and enzyme values differed <10% regarding independent transformants. FBPase activity was measured according to Gancedo (1971), PEPCK activity according to Hansen *et al.* (1976), and ICL activity according to Dixon and Kornberg (1959).

Gel retardation assays

Gel retardation experiments were performed as previously described (Niederacher *et al.*, 1992) with some minor variations. Binding reactions were carried out in GN buffer (30 mM Tris–Cl pH 7.5, 90 mM KCl, 7 mM $MgCl_2$, 1 mM DTT, 10 μM zinc acetate, 6% glycerol) with 1 μg of poly[dIdC], 30 μg of total cell protein and 20 000 c.p.m. of [^{32}P]dATP end-labelled oligonucleotides. The following synthetic oligonucleotides were used for gel retardation assays (original *PCK1* sequences are italicized, other nucleotides were added for cloning and labelling purposes): OMP31/32 (*PCK1* upstream region, positions –574/–542), 5′-AAT*TCGGGACGCTGACGGGTGAATGGAGATCTGGAT*-3′ and OMP33/34 (*PCK1* upstream region, positions –491/–460), 5′-AATT*AA-CCGAGCTTCCTTTCATCCGGCGCGGCTGTG*-3′; OMP1/2 5′-CTA-GACATTATGGATAGGCGGATAAAGGGCCCAAT-3′ for unspecific competition experiments.

Nucleotide sequence accession number

The GenBank/EMBL accession number for the *CAT5* sequence reported in this paper is X82930.

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