

Transcriptional regulation of *Saccharomyces cerevisiae* *CYS3* encoding cystathionine γ -lyase

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Received: 30 September 2007 / Revised: 29 January 2008 / Accepted: 9 February 2008 / Published online: 4 March 2008
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Abstract In studying the regulation of *GSH11*, the structural gene of the high-affinity glutathione transporter (GSH-P1) in *Saccharomyces cerevisiae*, a *cis*-acting cysteine responsive element, CCGCCACAC (CCG motif), was detected. Like GSH-P1, the cystathionine γ -lyase encoded by *CYS3* is induced by sulfur starvation and repressed by addition of cysteine to the growth medium. We detected a CCG motif (−311 to −303) and a CGC motif (CGCCACAC; −193 to −186), which is one base shorter than the CCG motif, in the 5′-upstream region of *CYS3*. One copy of the centromere determining element 1, CDE1 (TCACGTGA; −217 to −210), being responsible for regulation of the sulfate assimilation pathway genes, was also detected. We tested the roles of these three elements in the regulation of *CYS3*. Using a *lacZ*-reporter assay system, we found that the CCG/CGC motif is required for activation of *CYS3*, as well as for its repression by cysteine. In contrast,

the CDE1 motif was responsible for only activation of *CYS3*. We also found that two transcription factors, Met4 and VDE, are responsible for activation of *CYS3* through the CCG/CGC and CDE1 motifs. These observations suggest a dual regulation of *CYS3* by factors that interact with the CDE1 motif and the CCG/CGC motifs.

Keywords *CYS3* · Cystathionine γ -lyase · Regulatory motifs · Sulfur metabolism · *Saccharomyces cerevisiae*

Introduction

Genetic control of methionine biosynthesis has long been of interest (Thomas and Surdin-Kerjan 1997). Researchers in the de Robichon-Szulmajster Laboratory conducted an intensive study in *Saccharomyces cerevisiae* of methionine biosynthesis (Masselot and de Robichon-Szulmajster 1975), but its relationship to cysteine biosynthesis has remained obscure. *S. cerevisiae* can grow using methionine, homocysteine, cysteine, or glutathione as the sole sulfur source (Ono et al. 1984, 1988; Cherest and Surdin-Kerjan 1992). Subsequent work has determined that *S. cerevisiae* can reduce sulfate to form sulfide which is utilized to synthesize homocysteine (the sulfate assimilation pathway), and homocysteine-S is then converted to cysteine-S through cystathionine-S (the reverse trans-sulfuration pathway) (Ono et al. 1999). The reverse trans-sulfuration pathway consists of two enzymes, cystathionine β -synthase (β CTSase; EC 4.2.1.21) encoded by *CYS4* and cystathionine γ -lyase (γ CTLase; EC 4.4.1.1) encoded by *CYS3*.

In addition, we identified a high-affinity glutathione transport system (GSH-P1) encoded by *GSH11/HGT1/OPT1* in *S. cerevisiae* (Bourbouloux et al. 2000; Hauser

Communicated by K. Kuchler.

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et al. 2000; Miyake et al. 1998), and demonstrated that *GSH11* is induced by sulfur starvation and repressed by addition of cysteine to the culture medium through a 5'-upstream sequence (CCGCCACAC), hereafter referred as the CCG motif (Miyake et al. 2002). In the course of that study, we identified VDE and seven additional proteins that bind to the CCG motif. The homing endonuclease VDE, which is produced by processing of the *TFP1/VMA1* gene product (Hirata et al. 1990; Kane et al. 1990), is thought to function as a transcriptional activator of *GSH11* because *GSH11* is not expressed in a VDE-deleted strain, and the observed inability to express *GSH11* is overcome by the introduction of the coding region of VDE or the entire *VMA1* gene (Miyake et al. 2003). Given the observation that the activity of γ CTLase increases markedly under sulfur starvation and decreases markedly when cells are grown in the presence of organic sulfur compounds such as glutathione, methionine, and cysteine (Ono et al. 1991), we decided to investigate the regulation of *CYS3*.

In examining the 5'-upstream region of *CYS3*, we detected a CCG motif (CCGCCACAC) together with a CGCCACAC sequence, which is one nucleotide shorter than the CCG motif, and hereafter referred to as the CGC motif. In addition, a CDE1 motif (TCACGTGA) which is found in centromeres and in the 5'-upstream regions of genes encoding enzymes of the sulfate assimilation pathway (Barton et al. 1993; Kuras and Thomas 1995) was detected. A helix–loop–helix protein encoded by *CBF1* binds to the CDE1 motif and forms a heteromeric complex with Met4 and Met28 at the *MET16* promoter (Kuras and Thomas 1996). In addition, a *cbf1* null mutant displays methionine auxotrophy (Baker and Masison 1990). Genes in the sulfate assimilation pathway are regulated coordinately and *MET4* is indispensable for their expression (Thomas and Surdin-Kerjan 1997). Given the presence of these three putative regulatory elements, we hypothesized that *CYS3* would exhibit regulatory features similar to the sulfate assimilation pathway genes on the one hand and to *GSH11/HGT1/OPT1* on the other. In this study, we focused on the following three putative regulatory motifs in *CYS3*: CCG (CCGCCACAC, –311 to –303), CDE1 (TCACGTGA, –217 to –210) and CGC (CGCCACAC, –193 to –186) (Fig. 1). Our results reveal the complex regulatory control of *S. cerevisiae CYS3*, and we discuss our findings in relation to *S. cerevisiae* sulfur metabolism.

Materials and methods

Strains and plasmids

The *S. cerevisiae* strains used in this study were SF1-1C (*MAT α leu2 trp1 met17*) and YPH500 (*MAT α ade2 lys2 his3 leu2 trp1 ura3*), which are wild-type with respect to

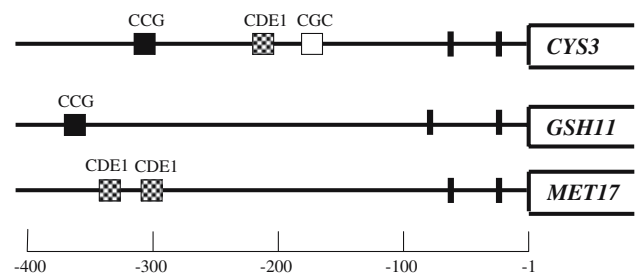


Fig. 1 Comparison of the 5'-upstream regions of *CYS3*, *GSH11*, and *MET17*. The cysteine-responsive element (–364 to –356; CCG) of *GSH11*, and the two regulatory centromere determining elements (–326 to –319, –301 to –294; CDE1) of *MET17*. The three putative regulatory motifs of *CYS3* are shown as CCG (–311 to –303), centromere determining element (–217 to –210; CDE1), and CGC (–193 to –186), respectively

γ CTLase activity and regulation. In the YOC2176 [*MAT α ade2 lys2 his3 leu2 trp1 ura3 VMA1-101 (vde-delta)*] strain, the VDE region has been disrupted (Miyake et al. 2003) and we constructed the YPH500m4 (*MAT α ade2 lys2 his3 leu2 trp1 ura3 met4::CgHIS3*) strain, by disrupting *MET4* in the YPH500 strain. The *Escherichia coli* strain DH10B was used for amplification of plasmids. We used p69-2-1 (Ono et al. 1992) as a template for PCR amplification of *CYS3*, pBluescriptII SK+ (TOYOBO, Osaka, Japan) for sequencing, pMC1587 (containing the *lacZ* coding region (Casadaban et al. 1983)) for analysis of the *CYS3* promoter region and pCgHIS3 (supplied by Dr. Harashima) for disruption of the *MET4* gene.

Growth media and growth conditions

We used standard yeast growth media (Sherman et al. 1986) such as YPD (1% yeast extract, 2% peptone, and 2% glucose) and synthetic minimal (SD) medium. Sulfur-free (SF) medium was prepared by replacing the sulfate salts in the SD medium (Wickerham 1956) with the corresponding chloride salts (Ono et al. 1991). Where appropriate, supplements were added to the SD and SF media. For example, MET and CYS media were prepared by the addition of 100 μ M methionine and 100 μ M (derepression) or 10 mM (repression) cysteine. *Escherichia coli* was cultivated in LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2) (Sambrook et al. 1989), with the addition of 50 μ g/ml ampicillin when required. Solid media contained 2% agar. *Saccharomyces cerevisiae* and *E. coli* were grown at 30 and 37°C, respectively, and liquid cultures were incubated on a reciprocal shaker at 120 rpm.

DNA manipulations

DNA manipulation (Sambrook et al. 1989) and transformation of *S. cerevisiae* (Ito et al. 1983) were performed as described previously.

Disruption of *MET4*

A DNA fragment containing the *Candida glabrata* *HIS3* gene was amplified by PCR from pCgHIS3 using the primers: 5'-AGT CCC ACG AAG GCG ACT CAT ACA GCA CGG AAT TCA TAA AGT TGT AAA ACG ACG GCC AGT-3' and 5'-ATG GAG CTT AGA AAA GAA GCC TCT GCT ACT ACA CCG TGC TCA CAG GAA ACA GCT ATG ACC-3'. The italicized sequences correspond to 10 bp downstream from the *MET4* ATG initiation codon and 951 bp upstream from the TAG termination codon, respectively. PCR-amplification was performed for one cycle at 94°C for 1 min, 56°C for 30 s, and 1 min at 72°C, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 1 min at 72°C, with a final extension step at 72°C for 7 min. The YPH500 strain was transformed with the 1.78 kb PCR product and His⁺ Met⁻ transformants were obtained. We confirmed that the transformant YPH500m4 was *met4*⁻ by complementation with the *MET4*-bearing plasmid pYCM4 (provided by Dr. Omura, Institute for Liquor Products, Suntory Ltd.).

Electrophoretic mobility shift assays

Saccharomyces cerevisiae SF1-1C was grown overnight in YPD and then transferred to either SF or fresh YPD media. After a 17 h incubation, the cells were harvested by centrifugation, washed once with homogenization buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine-HCl) and resuspended in 0.3 ml of the same buffer with 0.4 ml glass beads. The cell suspension was subjected to ten cycles of shaking for 30 s and cooling in an ice bath for 60 s; cell debris was then removed by centrifugation. The supernatant was used as the cell extract.

The mobility shift assays were performed as described previously (Miyake et al. 2002). In brief, DNA-protein binding reactions used cell extract (20 µg of protein) and digoxigenin-11-ddUTP (DIG)-labeled DNA probes (100 fmol) in a total volume of 10 µl containing 10 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 1% (w/v) Tween 20, 1 µg poly(dI-dC) and 30 mM KCl. Reactions were performed at 25°C for 30 min and terminated by adding 2 µl of loading buffer containing 0.2× TBE buffer and 40% glycerol. Fragments-1, -2, -3, -6, -7, and -8 were produced by PCR using the primers listed in Table 1 and end-labeled with DIG using terminal transferase, according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Two DIG-labeled fragments (F-4 and F-5) were custom-synthesized (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Using 2% agarose gels that had been pre-run (2 h) in 0.5× TBE buffer, terminated reaction mixtures were

Table 1 Primers used for amplification of the 5'-upstream fragments of *CYS3*

Primers	Sequence (5'→3')
USC3-1	AAT ACC CGG GTT CCA CCA GTG GGC CCA GTG
USC3-2	AAT ACC CGG GAG TGA CCG CCA CAC TGG ACC
USC3-3	AAT ACC CGG GTG GAC CCC ATA CCA CTT CTT
USC3-4	AAT ACC CGG GTA ACG CTA TGT AAT TCC ACC
USC3-5	AAT ACC CGG GAT TCA CGT GAT CTC AGC CAG
USC3-6	AAT ACC CGG GAG TAC AAT TGA GGC CTA TAC
USC3-7	AAT ACC CGG GCG CCA CAC TTT TTT TTC C
USC3-8	AAT ACC CGG GTT TTT TTT CCA TAA AAA T
USC3-9	AAT ACC CGG GCC GCC ACA CTT TTT TTT CC
USC3-10	CTG GCT GAG ATC ACG TGA AT
USC3-11	GGT GGA ATT ACA TAG CGT TA
USC3-12	TAA TTA TTA GTA ATG AAG GG

USC3-rev 5'-CAC AGG ATC CTT GGT AGC AAA TTT ATC AGA-3' was used as the reverse primer in PCR amplifications except those for internal deletion mutants (F23, F22 and F20), where USC3-10 (for F23 and F22) and USC3-11 (for F20) were the reverse primers, respectively; restriction sites are italicized, i.e., CC CGG G (*Sma*I) and GG ATC C (*Bam*HI).

subjected to electrophoresis for 2 h at 4°C, and then blotted onto nylon membranes (Amersham Pharmacia Biotech) by capillary transfer. DIG-labeled DNA was detected using an immunoassay with NBT/BCIP as the color-developing substrate, according to the manufacturer's protocol (Roche Diagnostics).

Competition assays

Competition experiments were performed as described above, with the addition of 100 pmol unlabeled oligonucleotides as the competitor. Four unlabeled double stranded fragments (URM-1, URM-2, URM-3, and URM-4) were custom-synthesized (Amersham Pharmacia Biotech).

Construction of plasmids for the *lacZ* reporter assay

Using p69-2-1 and the primers listed in Table 1, we PCR-amplified various fragments containing different segments of the 5'-upstream region of *CYS3*. The nucleotide sequence of the reverse primer (USC3-rev) encoded 12 amino acids from the N terminus of γ CTLase and a *Bam*HI site was designed near the 5' end. This reverse primer was used in conjunction with forward primers to various segments of the 5'-upstream region of *CYS3*. Forward primers contained a *Sma*I site at the 5' end. Although most PCR fragments were digested with *Sma*I and *Bam*HI, and then ligated into the *Sma*I/*Bam*HI site of pMC1587, fragments F20, F22 and F23 were digested with *Sma*I and ligated into

the *Sma*I site in the F19 plasmid (Fig. 5). All gene constructs encoded proteins in which the first eight amino acids of β -galactosidase were replaced by the first 12 amino acids of γ CTLase.

Assay for β -galactosidase activity

Cells were incubated in CYS (SF medium supplemented with 10 mM cysteine) or SF media for 17 h, then harvested and resuspended in 1 ml of Z buffer (Sherman et al. 1986), followed by treatment with 0.05 ml chloroform and 0.05 ml 0.1% SDS. β -galactosidase activity was measured at 30°C as the mean rate of hydrolysis of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) to *o*-nitrophenol (ONP) and galactose. β -galactosidase activity was calculated using the formula $A_{420} \times 1,000 \text{ min}^{-1} \text{ ml}^{-1} \text{ OD}_{600}^{-1}$.

Results

Electrophoretic mobility shift assays using segments from the 5'-upstream region of *CYS3*

To determine whether the transcriptional activators VDE and Met4 bind to the 5'-upstream region of *CYS3*, a 368-bp DNA fragment containing the three putative binding motifs CCG, CDE1, and CGC (Fig. 2a) was used as a probe (fragment F-1) in electrophoretic mobility shift assays. The probe DNA was mixed with cell extracts prepared from a wild-type strain (YPH500), a *met4* mutant (*met4::CgHIS3*), or a *vde*-delta strain. In order to examine the effects of sulfur starvation, the strains were grown in the presence

(YPD and SD) or absence (SF) of sulfate. As shown in Fig. 2b, regardless of the presence or absence of sulfate in the growth medium, all strains showed a slow-migrating band, indicating that all strains constitutively produced protein(s) that bound to the probe. However, the electrophoretic mobility of the band differed markedly between SD medium and SF medium for the wild-type and Δ *met4* strains, whereas such differences were not observed for the Δ *vde* strain. This result is consistent with our previous observation that γ CTLase activity increases in cells grown in SF medium but not YPD or SD medium (Ono et al. 1991).

Next, various segments of the 5'-upstream region of *CYS3* were PCR-amplified (Fig. 3a) and subjected to electrophoretic mobility shift assays in the presence of extracts prepared from wild-type cells grown in SF medium (Fig. 3b). The fragments F-2 (−73 to +36) and F-3 (−185 to +36) containing one and two TATA boxes, respectively, exhibited substantial band shifts in the presence of the cell extract, consistent with previous observations that *S. cerevisiae* possesses protein(s) that binds to the TATA box including the TATA-binding protein (TBP) (Buratowski et al. 1988). The fragment F-4 (−203 to −164), which contained a CGC motif, exhibited a marked band shift. The fragment F-5 (−233 to −194), which contained a CDE1 motif, revealed more than one shifted band, indicating a complex interaction of this fragment with different proteins. The fragments F-6 (−302 to −223) and F-7 (−302 to −241), which correspond to intervening segments between the CCG and CDE1 motifs, showed substantial band shifts, indicating the presence of protein-binding site(s) in this region. The fragment F-8

Fig. 2 Electrophoretic mobility shift assay using cell extracts from cells grown under various conditions. **a** The fragment indicated (*F-1*) was amplified using the primers listed in Table 1 (USC3-1 and USC3-rev). PCR product was end-labeled with DIG using terminal transferase. **b** The DIG end-labeled DNA fragment containing all three putative motifs (CCG, CDE1, and CGC) was added to the reaction mixtures in the presence of cell extracts from three kinds of strains [YPH500 (wild-type), YPH500m4 (*met4::CgHIS3*), and YOC2176 (*vde*-delta)] grown in the presence (YPD and SD) or absence of sulfur (SFree)

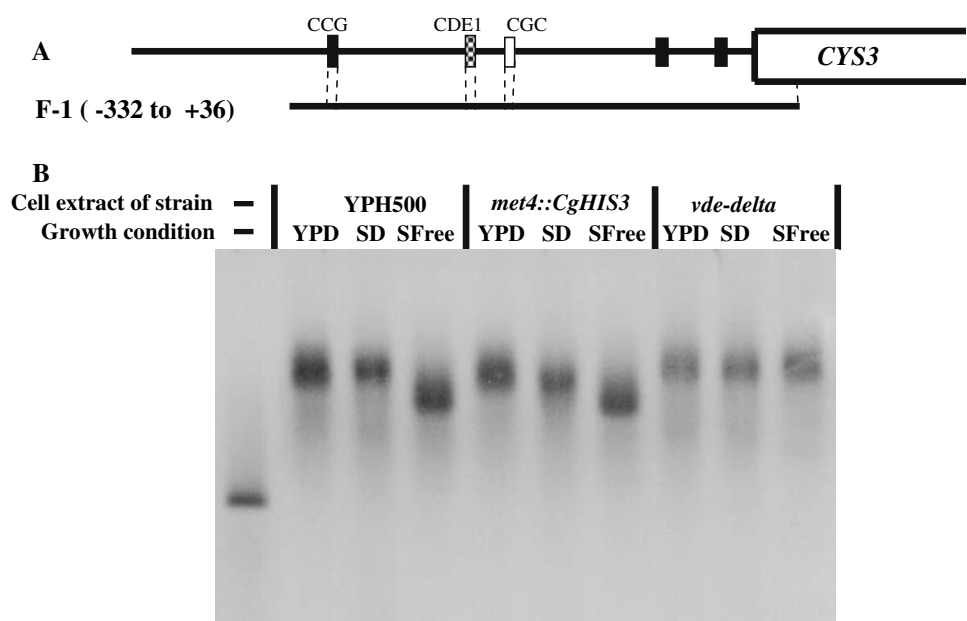
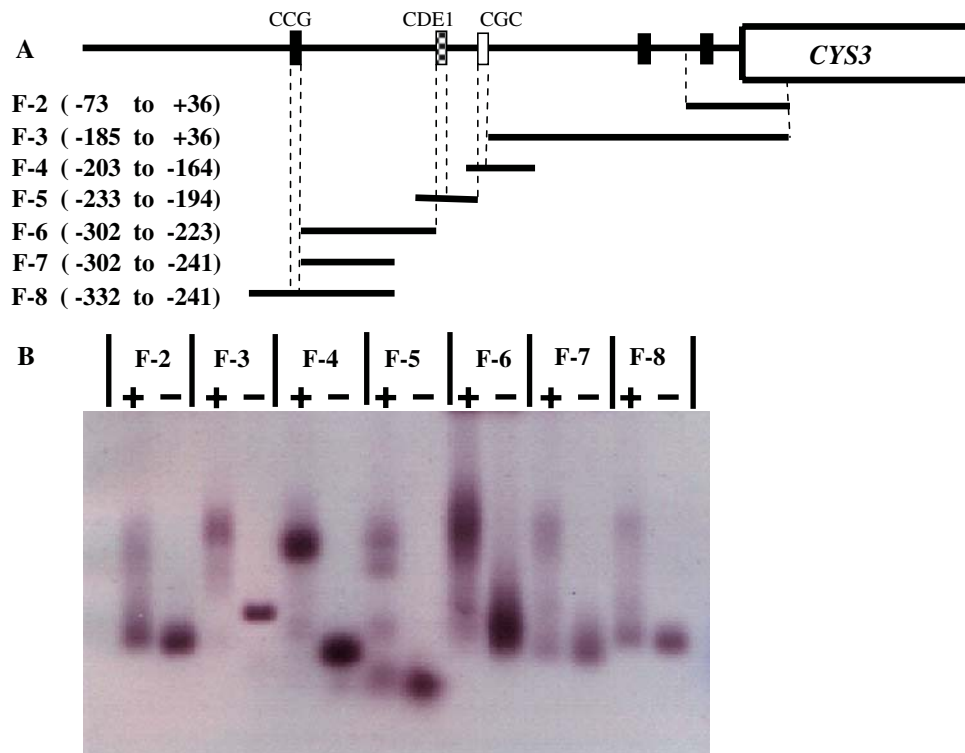


Fig. 3 Electrophoretic mobility shift assay. **a** The five fragments indicated (F-2, -3, -6, -7, -8) were amplified using the primers listed in Table 1 (F-2: USC3-6 and USC3-rev; F-3: USC3-8 and USC3-rev; F-4: USC3-3 and USC3-12; F-5: USC3-3 and USC3-11; and F-8: USC3-1 and USC3-11). PCR products were end-labeled with DIG using terminal transferase. The two DIG end-labeled DNA fragments indicated (F-4 and F-5) were custom-synthesized. **b** The DIG end-labeled DNA fragments containing consensus sequences from the *CYS3* promoter region were added to the reaction mixtures in the presence (+) or absence (–) of SFree-grown cell extract. The location of the putative regulatory motifs are as shown in Fig. 1



(–332 to –241) corresponding to F-7 plus the CCG motif exhibited a weaker band shift than the F-7 fragment. This result indicates that the binding of protein(s) to the CCG motif is to some extent inhibited by the presence of the neighboring nucleotide sequence. Taken together, these results show that *S. cerevisiae* grown under sulfur starvation conditions expresses a number of proteins that bind to the three motifs of interest.

In order to investigate the protein-binding sites in the F-4 (CGC motif) and F-5 (CDE1 motif) fragments, we performed competition assays using different sequences from the 5' upstream region of *CYS3* (Fig. 4a). URM-1 (–219 to –170; lane 3) and URM-3 (–193 to –174; lane 5) caused marked suppression of the band-shift of F-4, whereas URM-2 (–203 to –184; lane 4) and URM-4 (–185 to –170; lane 6) had no effect (Fig. 4b). We therefore concluded that protein(s) bind not to the CGC motif alone but to the CGC motif plus its 3' downstream sequence. In contrast, URM-1 and URM-2 were highly and only slightly suppressive, respectively, to proteins binding the F-5 fragment, suggesting that protein(s) bind to the CDE1 motif alone.

lacZ reporter assays

γ CTLase activity increases when cells are grown in SF medium (Ono et al. 1991). We therefore decided to determine the regulatory motifs involved in transcriptional activation of *CYS3*, under sulfur starvation conditions, with

a *lacZ* reporter assay using 5'-upstream segments of *CYS3*. We constructed plasmids in which various 5'-upstream segments of *CYS3* were inserted in front of *lacZ* (Fig. 5). The constructs were then introduced into the YPH500 strain (wild-type), and β -galactosidase activity was measured (Table 2, 1st column). Although 100 μ M cysteine in the growth medium caused a decrease in γ CTLase activity in our previous study (Ono et al. 1991), 100 μ M or even 1 mM cysteine, did not cause a decrease in β -galactosidase activity. The F07 construct exhibited β -galactosidase activity of 611.4 U min⁻¹ OD₆₀₀⁻¹ in the presence of 1 mM cysteine. However, 10 mM cysteine caused an obvious decrease in β -galactosidase activity. We attribute the observed difference in the effective cysteine concentration to the inhibitory effect of cysteine on γ CTLase but not on β -galactosidase. Therefore, we used 10 mM cysteine for the subsequent studies. In the absence of cysteine, the F07 construct (containing all three motifs of interest) exhibited β -galactosidase activity of 617.4 U min⁻¹ OD₆₀₀⁻¹, whereas in the presence of 10 mM cysteine, the activity level was 309.1 U min⁻¹ OD₆₀₀⁻¹, indicating that cysteine caused a 50% decrease in gene expression relative to the derepressed level. The F17 construct (containing one TATA box) exhibited a β -galactosidase activity of 2.9 U min⁻¹ OD₆₀₀⁻¹ regardless of the presence or absence of cysteine. The F19 (containing two TATA boxes) and F22 (containing the CDE1 motif in addition to two TATA boxes) constructs exhibited approximately ninefold and 81-fold the activity, respectively, of the F17 construct regardless of the presence

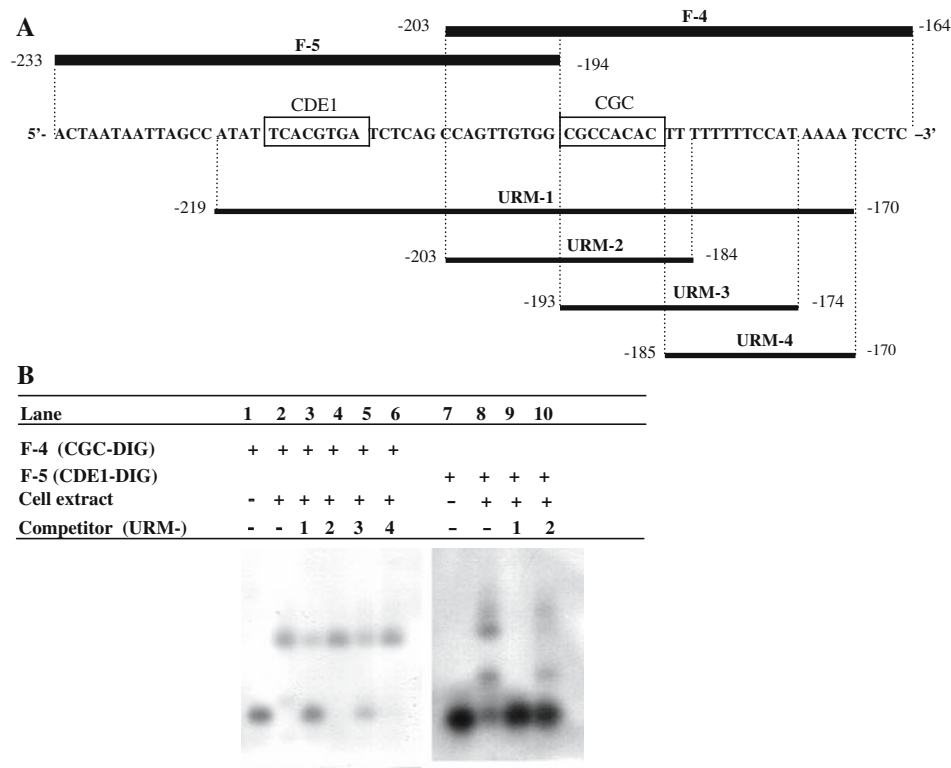


Fig. 4 Competition assay. **a** The six custom-synthesized fragments indicated [*URM1–URM4*, DIG end-labeled *F-4* (*CGC*), and DIG end-labeled *F-5* (*CDE1*)] correspond to different segments of the 5'-upstream region of *CYS3*. The fragments corresponding to -203 to -164 (*CGC*) and -233 to -194 (*CDE1*) were used as the target DNA and the other fragments were used as competitors. The locations of the putative regulatory motifs are as shown in Fig. 1. **b** In the left panel, the target *CGC* fragment was incubated alone or with cell

extract (*lanes 1* or *2*) in the absence of competitor, or in the presence of a 1,000-fold excess of unlabeled double stranded *URM1* (*lane 3*), *URM2* (*lane 4*), *URM3* (*lane 5*), or *URM4* (*lane 6*). In the right panel, the target *CDE1* fragment was incubated alone or with cell extract (*lanes 7* or *8*) in the absence of competitor, or in the presence (*lanes 9* and *10*) of a 1,000-fold excess of the unlabeled competitor as indicated

or absence of cysteine. However, the fold induction by *F22* was approximately the same as by *F19* and *F17*. Therefore, we concluded that the *CDE1* element is involved in constitutive expression of the target gene but not in sulfur starvation-dependent expression.

In contrast, the *F18* construct (containing the *CGC* motif in addition to the two TATA boxes) exhibited 80% the activity of the *F07* construct in the absence of cysteine, and 30% the activity of *F07* in the presence of 10 mM cysteine, indicating that the *CGC* motif is responsible for strong activation in the absence of cysteine, and strong repression in its presence (5-fold induction). Since the *F21* construct (containing the *CCG* motif located in the position of the *CGC* motif) behaved similarly to the *F18* construct, it is possible that, in closer proximity to the coding region, the *CCG* motif can function in a similar manner to the *CGC* motif (2.9-fold induction). The *F20* construct (containing the *CCG* motif and its adjacent downstream regions) exhibited approximately half the activity of the *F21* construct in the absence of cysteine. Notably, the *F20* construct contains not only the *CCG* motif but also the adjacent regions. Therefore, we concluded that either the

sequence(s) adjacent to the *CCG* motif are inhibitory or the distance between the motif and its target gene is critical for the activator function of the *CCG* motif.

As observed in comparison of the *F10*, *F08*, and *F23* constructs, the presence of the *CDE1* motif plus either the *CGC* motif or the *CCG* motif caused a high level of constitutive expression of the target gene. In contrast, the *CCG* and *CGC* motifs alone are responsible for a high level of inducible expression.

Involvement of the homing nuclease VDE in the regulation of *CYS3*

To ascertain the involvement of VDE in regulation of *CYS3*, *lacZ* reporter constructs were introduced into the YOC2176 strain (a *vde*-delta derivative of YPH500; Table 2, 2nd column). In the absence of cysteine, *F07* (containing all three motifs of interest) exhibited somewhat lower activity in the Δvde mutant (YOC2176) than in the wild-type strain (YPH500); that is, the fold induction by *F07* was 2 \times for the wild-type strain and 1.5 \times for the Δvde mutant. In the presence of cysteine, there was no detectable

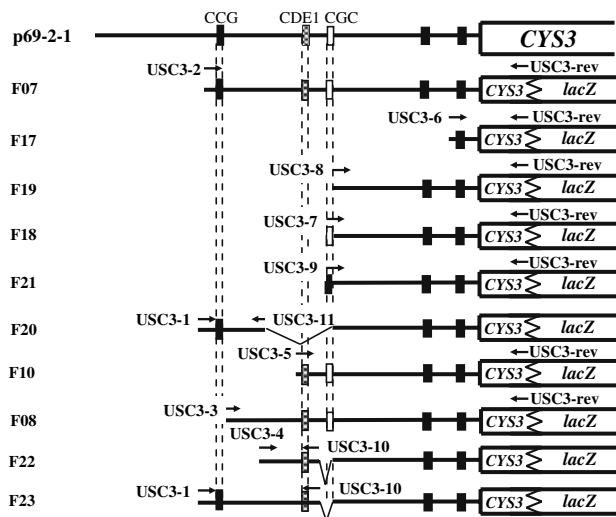


Fig. 5 Plasmids used for the *lacZ* reporter assay. In order to construct plasmids containing sequential deletions, we PCR-amplified various DNA fragments from *p69-2-1* using the primers indicated. These fragments contained different combinations of the *CCG*, *CDE1*, and *CGC* motifs. The PCR products were digested with *Sma*I and *Bam*HI, then ligated into the *Sma*I/*Bam*HI site of pMC1587. The internal deletion plasmids containing CCG and/or CDE1 were generated by PCR amplification using Pyrobest DNA polymerase (Takara, Shiga, Japan). The fragments *F20* (CCG motif), *F22* (CDE1 motif) and *F23* (CCG and CDE1 motifs) were digested with *Sma*I, and ligated into the *Sma*I site of *F19*

difference between the two strains. A more significant difference was observed for the *F18* (CGC motif) and *F21* (CCG motif) constructs. In the absence of cysteine, both constructs exhibited approximately one-third the activity in the Δvde mutant as in the wild-type strain. In contrast, there was no detectable difference between the strains in the presence of cysteine. *F22* (CDE1 motif) caused slightly lower gene expression in the Δvde mutant, relative to the wild-type strain, in the absence of cysteine. *F10* showed approximately two-thirds the level of gene expression in the Δvde mutant relative to the wild-type strain in the absence of cysteine, while both strains exhibited similar levels of gene expression in the presence of 10 mM cysteine. Therefore, VDE may play a role in activation of *CYS3*. In the absence of cysteine, similar β -galactosidase activities were observed with the *F07*, *F10*, *F08*, and *F23* constructs. The latter three constructs contain the CDE1 motif plus either the CGC (*F10* and *F08*) or CCG motif (*F23*); the level of gene expression due to these constructs was reduced by the addition of cysteine. In contrast, the *F18* (CGC motif alone), *F20* and *F21* (CCG motif alone), and *F22* (CDE1 motif alone) constructs induced intermediate levels of gene expression, and the expression level was not affected by the addition of cysteine. Hence, the expression of *CYS3* via the CGC motif or the CCG motif is weaker in the Δvde mutant than in the wild-type strain.

These results suggest the following conclusions: (1) the CGC and CCG motifs are equally effective for *CYS3* activation, and this activation is not subjected to repression by cysteine; (2) the CDE1 motif alone exhibits a level of activation similar to the CGC and CCG motifs; and (3) the CGC and CCG motifs increase activation in the presence of the CDE1 motif, and this activation is subject to repression by cysteine. The wild-type strain (YPH500) and the *vde*-delta strain (YOC2176) exhibited a marked difference in activation mediated by the CGC (*F18*) or CCG (*F21*) motif and by combining the CDE1 motif with either the CGC (*F10* and *F08*) or CCG (*F23*) motif. Based on the present observations, together with our previous finding that VDE binds the CCG motif (Miyake et al. 2003), we contend that VDE plays a role in activation of *CYS3* via the CGC and CCG motifs.

Role of *MET4* in the regulation of *CYS3*

In order to investigate the role of *MET4* in regulation of *CYS3*, we constructed the *met4* mutant YPH500m4 and performed *lacZ* reporter assays using this strain (Table 2, the 3rd column). In all assays, the level of β -galactosidase activity was low, suggesting that *MET4* is required for regulation mediated by each of the three motifs. However, the *F22* construct (CDE1 motif alone) exhibited a higher level of induction in the *met4* mutant (YPH500m4) than in the wild-type strain (YPH500); that is, the fold induction for construct *F22* was 0.9 \times for the wild-type strain and 1.9 \times for the *met4* mutant. This result suggests that an auxiliary transcription factor other than *Met4* is involved in expression of *CYS3* under sulfur starvation conditions.

Discussion

In this study, we examined the roles of three putative regulatory motifs present in the 5'-upstream region of the *S. cerevisiae* *CYS3* gene, CGC (−193 to −186), CDE1 (−217 to −210), and CCG (−311 to −303). We found that the CCG and CGC motifs play roles in expression of the target gene and that differences observed between the motifs are largely attributable to their proximity to the target gene. As the CGC motif (CGCCACAC) is only one nucleotide shorter than the CCG motif (CCGCCACAC), we suggest that the functional body of the motifs is identical and should be referred to as the CCG/CGC motif. Activation by the CCG/CGC motif is repressed by 10 mM cysteine, and this response is lowered substantially in the absence of VDE, suggesting that VDE binds directly or indirectly to the CCG/CGC motif to activate the target gene. We have also found that a high concentration of cysteine (10 mM) is

Table 2 Analysis of 5'-upstream region of *CYS3*

5'-upstream segment	β -Galactosidase specific activities ($\text{U min}^{-1} \text{OD}_{600}^{-1}$)											
	Wild-type						<i>ude-delta</i>			<i>met4::CgHIS3</i>		
	0 mM Cys (D)	10 mM Cys (R)	(D/R)	0 mM Cys (D)	10 mM Cys (R)	(D/R)	0 mM Cys (D)	10 mM Cys (R)	(D/R)	0 mM Cys (D)	10 mM Cys (R)	(D/R)
F07	617.4 \pm 116	309.1 \pm 54	2	446.3 \pm 26	292.4 \pm 71	1.5	42.7 \pm 6.2	29.5 \pm 8.3	1.4			
F17	2.9 \pm 0.2	2.8 \pm 0.9	1	nd	nd	–	nd	nd	–			
F19	26.2 \pm 7.7	26.0 \pm 1.4	1	31.2 \pm 6.6	17.4 \pm 5.3	1.8	10.4 \pm 2.7	17.3 \pm 1.2	0.6			
F18	522.6 \pm 40	97.1 \pm 12	5.4	166.8 \pm 22	121.0 \pm 29	1.4	1.5 \pm 3.6	18.0 \pm 3.0	0.6			
F21	429.4 \pm 42	146.4 \pm 46	2.9	161.1 \pm 16	120.2 \pm 27	1.3	36.5 \pm 6.5	25.2 \pm 5.6	1.4			
F20	241.1 \pm 41	181.1 \pm 34	1.3	167.5 \pm 29	197.2 \pm 37	0.8	97.1 \pm 1.5	71.7 \pm 4.0	1.4			
F10	586.6 \pm 61	216.4 \pm 47	2.7	386.3 \pm 76	278.9 \pm 55	1.4	28.5 \pm 8.9	21.2 \pm 3.4	1.3			
F08	642.8 \pm 61	216.1 \pm 16	3	390.5 \pm 50	298.9 \pm 81	1.3	35.1 \pm 2.7	24.8 \pm 9.5	1.4			
F22	245.1 \pm 18	277.5 \pm 27	0.9	183.4 \pm 18	197.0 \pm 19	0.9	111.9 \pm 31	57.7 \pm 17	1.9			
F23	568.4 \pm 32	279.1 \pm 63	2	374.5 \pm 64	271.8 \pm 49	1.4	104.0 \pm 15	60.3 \pm 2.2	1.7			

Different segments of the 5'-upstream region of *CYS3* were PCR-amplified and fused to the *E. coli lacZ* in plasmid pMC1587. The constructs were transformed into the YPH500 (wild-type), YOC2176 (*ude-delta*) and YPH500m4 (*met4::CgHIS3*) strains and grown in SFree medium supplemented with 0 or 10 mM cysteine. β -galactosidase activities were measured for each culture and values represent means (\pm standard deviations) of three independent experiments. The locations of the putative regulatory motifs are as shown in Fig. 1 *nd* not determined

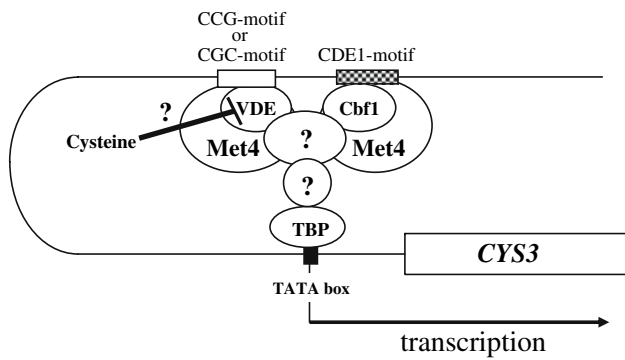


Fig. 6 A proposed model for regulation of *CYS3*. In the absence of cysteine, the transcription factor complexes *Met4*–*Cbf1* and/or *Met4*–*VDE* bind the *CDE1* or *CCG/CGC* motifs, respectively, then these complexes interact with basal transcription factors such as *TBP*, resulting in the activation of *CYS3* transcription. In the presence of cysteine, *CYS3* expression is repressed by the abundant cysteine molecules interacting directly or indirectly with *VDE*

dependence of the CCG/CGC motif. Furthermore, it is clear that *MET4* is required for activation via both the *CDE1* motif and the CCG/CGC motif. In view of these and earlier findings, we propose a model whereby *CYS3* is under dual regulation by the *CDE1* motif and the CCG/CGC motif (Fig. 6). We suggest that both the *Cbf1*–*Met4* and *VDE*–*Met4* complexes bind to the *CDE1* motif and the CCG/CGC motif, and that these two regulatory systems can operate independently as well as cooperatively. As binding of *TBP* to the TATA box serves as an anchor for RNA polymerase to bind DNA for the initiation of RNA synthesis (Buratowski et al. 1988), we hypothesize that *TBP* binds directly or indirectly to the *Cbf1*–*Met4* and *VDE*–*Met4* complexes.

In this investigation, we focused on the CCG/CGC and *CDE1* regulatory motifs. However, *CYS3* also possesses a *Met31*/*Met32* binding site (*MBS* motif; –198 to –193) (Blaiseau et al. 1997; Chiang et al. 2006). Our data (Table 2) indicate that the CCG/CGC motif regulates expression of *CYS3*, but the *MBS* motif does not. In the 5'-upstream region of *CYS3*, there are also regulatory motifs that appear to be involved in the stress response, such as the *yAP-1* response element (*YRE* motif; –235 to –229) (Lee et al. 1999) and the *Xbp1* binding site (*XBS* motif; –166 to –161) (Mai and Breeden 2000). Since glutathione plays a central role in the cellular response to oxidative stress and is synthesized using cysteine as a substrate, there appears to be a link between expression of *CYS3* and the roles of these stress response motifs. This line of investigation remains for future studies.

It is now clear that the CCG/CGC motif is responsible for regulation of both *GSH11* and *CYS3*. By searching the *S. cerevisiae* genome (<http://www.yeastgenome.org/>), we found that several ORFs have motifs identical or similar to the CCG/CGC motif in their 5'-upstream regions. While some are involved in sulfur metabolism, such as *SPE2* (encoding SAM decarboxylase, EC 4.1.1.50), *MET13* (encoding a putative 5,10-methylenetetrahydrofolate reductase, EC 1.5.1.20), and *MET2* (encoding homoserine *O*-acetyltransferase, EC 2.3.1.31) (Fig. 7), the functions of the others are not known. It will be of interest in future studies to determine whether or not these genes and ORFs are regulated via *Met4* and *VDE*.

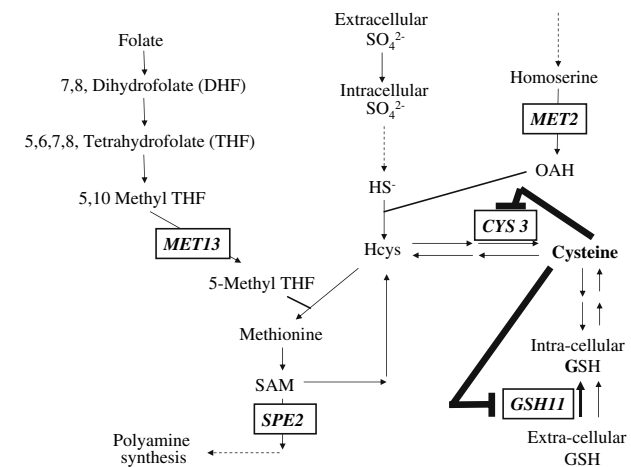


Fig. 7 Regulation of *S. cerevisiae* sulfur metabolism by cysteine. The boxed sulfur metabolism genes contain one or more CCG/CGC motif(s) in their 5'-upstream regions. *CYS3* and *GSH11* are regulated by the amount of available cysteine in the cell

necessary for repression of the *CYS3* gene. Our result is consistent with the results reported by Hansen and Johannesen (2000), in that cysteine repression of the sulfate assimilation pathway genes *MET14* and *MET25* occurred at a concentration of 10 mM cysteine. Thus, quite high levels of cysteine appear to be needed for repression of the *CYS3*, *MET14*, and *MET25* genes.

The *CDE1* motif is responsible for intermediate activation of the target gene, but not for repression by cysteine. Activation via the *CDE1* motif and via the CCG/CGC motif was additive, indicating that distinct activation complexes are interacting with these two kinds of motif. Activation due to the *CDE1* motif appears to be dependent upon *VDE*, though to a much lesser extent than the *VDE*

Acknowledgments This work was supported by funds from the Bio-Venture project and the 21st century COE program of Ritsumeikan University. We are grateful to S. Harashima (Osaka University) and F. Omura (Institute for Liquor Products, Suntory Ltd) for their kind gifts of plasmids containing *Candida glabrata* *HIS3* and *MET4*, respectively.

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