The *Saccharomyces cerevisiae* gene *HGT1/GSH11* encodes the high affinity glutathione transporter and is repressed by cysteine added to the culture medium. It has been found previously that a 5'-upstream *cis*-element, CCGCCACAC, is responsible for regulating *GSH11* expression and that several proteins bind to this element (Miyake, T., Kanayama, M., Sammoto, H., and Ono, B. (2002) Mol. Genet. Genomics 266, 1004-1011). In this report we present evidence that the most prominent of these proteins is VDE, known previously as the homing endonuclease encoded by *VMA1*. We show also that *GSH11* is not expressed in a VDE-deleted strain and that inability to express the *GSH11* of this strain is overcome by introduction of the coding region of VDE or the entire *VMA1* gene. It is also found that VDE does not cut DNA in the vicinity of the *GSH11* cis-*element*. Rapamycin, an inhibitor of the target of rapamycin (TOR) signal-transduction system, is found to enhance expression of *GSH11* in a VDE-dependent manner under conditions of sulfur starvation. These results indicate that *GSH11* is regulated by a system sensitive to sulfur starvation (presumably via cysteine depletion) and a more general system involving the nutritional starvation signal mediated by the TOR system. Both systems need to be operational (inhibition of TOR and sulfur starvation) for full expression of *GSH11*.

Glutathione plays essential roles in the detoxification of various toxic agents (2–5) including reactive oxygen species (6–8). Depletion of glutathione causes an increase of reactive oxygen species and results in death of the cell (9–11). Glutathione is the most abundant sulfur-containing compound in many organisms (12–14). It contains cysteine and produces cysteine by degradation. Thus, it acts as a reservoir of cysteine, thiol groups, and sulfur (15). Under conditions of sulfur starvation, glutathione is the last of various sulfur-containing compounds to decrease in the cell. It is also known that, under conditions of sulfur starvation, *Saccharomyces cerevisiae* utilizes sulfate, produced by the consumption of glutathione, as a sulfur source (16). Therefore, it appears that maintenance of the intracellular level of glutathione is of great importance for viability in terms of sustaining sulfur metabolism and protecting the cell from oxidative stress.

Because many organisms have the ability to utilize exogenous glutathione, we have focused our attention on the glutathione uptake activity of *S. cerevisiae* and have shown previously that this organism has two kinetically distinguishable glutathione transport systems, *i.e.* an inducible high affinity transporter (GSH-P1) and a constitutive low affinity one (GSH-P2) (17). GSH-P1 mediates transport of pentapeptides as well as enkephalin, but it should be mentioned that the affinity of GSH-P1 to the oligopeptides is much lower than that to glutathione (1). GSH-P1 has characteristics common to the OPT family of transporters present in fungi but not in mammals and bacteria; this is the reason why it is also referred to as OPT1 (18, 19). Recently, *Arabidopsis* has been shown to encode proteins belonging to this family (20). Glutathione and oligopeptides are convenient and useful sulfur sources if they are available in the environment. For this reason also, we are interested in the GSH-P1 transporter, particularly in the regulation of its production. We have already found that the gene coding for GSH-P1, *i.e.* *HGT1/GSH11*, has a novel *cis*-acting regulatory motif, CCGCCACAC, in its 5' upstream region (1).

In this study, we analyzed proteins that bound to the regulatory motif of *GSH11* and found that the most prominent was VDE, the homing endonuclease derived from *VMA1*. We investigated the role of VDE in the regulation of *GSH11* and also examined the effect of rapamycin, an inhibitor of the target of rapamycin (TOR) signal transduction system, on the regulation of *GSH11*.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The *S. cerevisiae* strains used in this study are listed in Table I. Strains SF1-1C and YPH500 are wild types in regard to the GSH-P1 activity and regulation of GSH-P1. They have the wild type allele in the *VMA1* locus. Strain YOC2176 is a gene-manipulated derivative of strain YPH500; it has the VMA1-101 (vde-delta) allele in which the whole VDE coding region is deleted from *VMA1* (21, 22). *Escherichia coli* strain JM109 (ThKaRa) was used throughout to amplify plasmids. pMC1587, a plasmid containing the coding region of locZ (23), was used for analysis of the promoter region of *HGT1/GSH11*. Plasmids YPYO314-VMA1 and YCY6TV-VDEc carry the wild type allele of *VMA1* and the entire coding region of VDE, respectively (21, 22).

Standard yeast growth media were used (24). YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose; SD medium is a synthetic minimal medium (25). Sulfur-free (SF) medium was prepared by substituting all sulfate salts in SD medium with the corresponding chloride salts (26). To provide for the nutritional requirements of the strains used in this study, adenine (20 μg/ml), lysine (30 μg/ml), histidine (20 μg/ml), leucine (30 μg/ml), tryptophan (20 μg/ml), and uracil...

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*The abbreviations used are: TOR, target of rapamycin; SF, sulfur-free; DIG, digoxigenin; DTT, dithiothreitol.*

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1 B-I. Ono, T. Hazu, and M. Kanayama, personal communication.
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(20 μg/ml) were added to the SD and SF media. To obtain a medium with a defined sulfur source, cysteine was added to SF medium at a concentration of 100 μM. Rapamycin (Sigma) was used at a final concentration of 200 nM. A 1 mg/ml stock solution of rapamycin was prepared in 90% ethanol and 10% Tween 20. For solid medium, 2% agar was added. The growth temperature was 30 °C, and liquid cultures were rotary shaken at 120 rpm.

LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2) was used for growth of E. coli, (27). Ampicillin was added to LB medium at a concentration of 50 μg/ml to screen for Amp\’ clones. For solid medium, 1.5% agar was added. The growth temperature was 37 °C, and liquid cultures were rotated rotary shaken at 120 rpm.

DNA Manipulations—Extraction of S. cerevisiae genomic DNA and transformation of S. cerevisiae were carried out as described (28, 29). Standard DNA manipulation procedures were adopted (27).

Polymerase Chain Reaction—Amplification of DNA fragment by PCR was carried out using TaKaRa ExTaq™ polymerase and a thermal cycler (Atto). The PCR program we adopted was as follows: step 1, denaturation at 93 °C for 1 min; step 2, 30 cycles of denaturation at 93 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and step 3, extension at 72 °C for 10 min. The PCR reaction mixture (50 μl) was prepared as described in the manufacturer’s instructions.

Identification of DNA-binding Proteins—Cells of strain SF1-1C were grown overnight in YPD medium and transferred to SF medium. After incubation for 16 h, they were harvested by centrifugation and washed once with homogenization buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine-HCl). The cells were resuspended in 0.3 ml of the same solution containing ce16-binding proteins; experimental details were included in the manufacturer’s instructions.

Two complementary 41-mer DNAs, corresponding to the cis-element of the GSH11 sequence from −371 to −331 (5’TTCGGCCCGCCACACCTCGGAC-TACAAGACGCCACATCTA-3’) containing the cis-element (underlined), were custom synthesized, annealed, and used as a double strand DNA probe, referred to as ce16. The probe was end-labeled with terminal transferase and digoxigenin (DIG)-11-dUTP according to the manufacturer’s instructions (Roche Diagnostics). Following this, the DIG-labeled DNA probe, ce16-DIG, was bound to anti-DIG magnetic particle as described by the manufacturer (Roche Diagnostics).

Binding reactions were carried out in a 200-μl binding buffer (10 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 1% (w/v) Tween 20, and 30 mM KCl) containing 7.5 pmol of ce16-DIG/anti-DIG particles and ~0.72 mg of cell extract protein. The binding reactions were incubated at 25 °C for 30 min. Complexes of magnetic particles and the DIG-DNA probe with bound proteins were separated with a magnetic separator and washed four times with binding buffer.

Binding proteins were eluted by successive incubation with a total of 30 μl (3 × 10 μl) of homogenization buffer containing KCl, the concentration of which was increased stepwise from 0.4 to 1 M. Aliquots of the eluted fractions were fractionated on SDS-PAGE (5–20% gradient gel, 64 V, ~3 h), and the gels were silver-stained for observation.

For identification of the protein bands, the binding reaction was scaled up to a total of 900 μl containing 105 pmol of magnetic particles and the DIG-DNA probe and ~3.9 mg of protein extract and concentrated by elution in a final volume of 20 μl. After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes by electroblotting (Nova-blot: Amersham Biosciences). The membranes were stained with Coomassie Brilliant Blue, and bands of interest were cut out for protein sequencing, which was carried out by APRO Life Science Institute, Inc., Naruto, Japan.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were carried out as described previously (1). DNA-protein binding was achieved at 25 °C for 30 min in a mixture (10 μl) containing 10 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 1% (w/v) Tween 20, 30 mM KCl, 0.8 ng of DIG-labeled DNA probe (ce16), and 1 μl of the solution containing ce16-binding proteins; experimental details can be found under the heading “Identification of DNA-binding Proteins” above.

Reporter Gene Assay—A DNA fragment corresponding to the segment from −371 to 33 in the 5′-upstream region of GSH11 was PCR-amplified using the genomic DNA of strain SF1-1C as template. The primers used were 5′-AATACCCCGGTTCGCCGCCACACCTCCGA-TCAAGACGCCACATCTA-3′ (forward) and 5′-CACAGGTCAACACGGTGTCGTCACCTCCAT-TAA-3′ (reverse). The PCR product was digested with Smal and BamHI and inserted into the Smal/BamHI site proximal to the coding region of lacZ in pMC1687 to generate plasmid EUG11-9; note that in the resulting fusion protein the first eight amino acids of the \( \beta \)-galactosidase were replaced with the first 11 amino acids of GSH11.

The structure of plasmid EUG11-9 was confirmed by sequencing, and EUG11-9 was used to transform strains YPH500, YOC2176, YOC2176/pYO314-VMA1, and YOC2176/YcpTV-VDEc. After incubation of the

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**Table I**

| Strain       | Genotype        | Source      |
|--------------|-----------------|-------------|
| SF1-1C       | MATα leu2 trp1 met17 | Miyake      |
| YPH500       | MATα ade2 lys2 his3 leu2 trp1 ura3 | ATCC     |
| YOC2176      | MATα ade2 lys2 his3 leu2 trp1 ura3 | Ohyya     |

* American Type Culture Collection, P. O. Box 1549, Manassas, VA 20108.

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Figs. 1. Detection of proteins bound to the cis-element of GSH11. Proteins binding to the cis-element of GSH11 were eluted successively with 0.4–1 M KCl solutions as indicated. Each fraction was separated by SDS-PAGE and silver-stained (see “Experimental Procedures”). At least eight bands are visible, the strongest of which is estimated to be 50 kDa by reference to molecular markers.
resultant transformants in SF medium or SF medium supplemented with 0.1 mM cysteine for 16 h, the cells were harvested and lysed. Then, the β-galactosidase activity was measured by mean of the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) to produce o-nitrophenol (ONP) and galactose. β-Galactosidase activity (in units) was calculated using the formula A420 × 1000/min/ml/OD600 at 37°C.

Northern Blot Hybridization—Strains YPH500 and YOC2176 were grown overnight in YPD medium. The cells were harvested and suspended in SF medium or SF medium supplemented with 0.1 mM cysteine. After incubation for 16 h, total RNA was extracted by the method of Elder et al. (30). RNA (20 μg per lane) was fractionated by agarose gel electrophoresis and subjected to Northern blot hybridization using a 741-bp fragment corresponding to a portion (T10–1450) of GSH11 as the probe (1).

VDE Cleavage—An 846-bp DNA fragment including the cis-element of GSH11 was PCR-amplified using genomic DNA of strain SF1-1C as template. Primers used were 5′-AATACCGGCTAGACCCATGTGTCGCAGGA-3′ (forward) and 5′-CAACCGAATCACGACTGGCCTCTTCCCTATAA-3′ (reverse). About 0.22 μg of the PCR product was incubated at 37 or 25 °C for 16 h with 2 units of purified VDE (New England Biolabs) dissolved in 20 μl of buffer (100 mM KCl, 10 mM Tris-Cl, 10 mM MgCl2, and 1 mM DTT, pH 8.6) supplemented with 100 μg/ml bovine serum albumin. The reaction was stopped by the addition of 0.5% SDS, and the samples were subjected to electrophoresis in 1× Tris acetate buffer with EDTA (TAE) on a 0.7% agarose gel. After electrophoresis, the gel was stained with SYBR Gold (Molecular Probes) and subjected to sequencing. From a homology search, the N-terminal sequence was matched with the VDE protein. * indicates refractory to analysis. Using a homology search we found that VDE, the homing endonuclease encoded by VMA1, has the identical N-terminal amino acid sequence (Fig. 2).

Involvement of VDE in Regulation of GSH11—To investigate the involvement of VDE in the regulation of GSH11, we conducted reporter gene assays using a strain in which the VDE coding region of VMA1 was deleted. For this experiment we constructed a plasmid in which the 5′-upstream region (from −371 to 33) of GSH11 was inserted in front of the coding region of the E. coli lacZ gene of the expression vector, pMC1587. Strains YPH500 (VMA1) and YOC2176 (a vde-delta derivative of YPH500) were transformed with the resultant plasmid, designated EUG11-9. Representative transformants were incubated for 16 h in SF medium or SF medium supplemented with 0.1 mM cysteine. The transformants derived from the VMA1 strain had higher β-galactosidase activity in sulfur starvation conditions and markedly lower activity in the presence of cysteine in the growth medium. In contrast, those derived from the vde-delta strain had very low β-galactosidase activity in both sulfur starvation and cysteine-supplemented conditions (Fig. 3).

We assayed the mRNA contents of the above mentioned cells by Northern blot hybridization using GSH11 as the probe (see “Experimental Procedures”). The VMA1 strain revealed a substantial level of expression of GSH11 in SF medium, whereas the vde-delta strain revealed no detectable level of expression (data not shown). Moreover, we found that the vde-delta strain recovered the ability of regulation of GSH11 if transformed with either plasmid pYO314-VMA1 (carrying the entire region of VMA1) or plasmid YCpTV-VDEc (carrying the entire VDE coding region). When the representative transformants were subjected to the reporter gene assay, they, like the VMA1 strain (YPH500), yielded substantial levels of β-galactosidase activity in sulfur starvation conditions (Fig. 3). All these results clearly indicate that VDE is involved in the expression of GSH11.

VDE Does Not Cut the GSH11 cis-Element—VDE is known as a homing endonuclease, but its target sequence remains obscure; that is, although single base substitutions reduced the susceptibility of target DNAs, none gained complete resistance to VDE (31, 32). The precise boundary of the target sequence is also not clear. Nevertheless, the following sequence is thought to be the most likely target of VDE (indicates cleavage site)

\[33': 5′-AATCTAAGCTTG GGGC CCCTCAACGCTGCAATGGCA-3′ \]

\[33': 3′-TAGATACAGCC TCGT TCCATTACTTTACCGT-5′ \]
Because the cis-element (CCGGCCACAC) of GSH11 (1) is somewhat similar to this sequence (underlined), we tested whether VDE binds to and cuts at the GSH11 cis-element. An 846-bp DNA fragment containing the GSH11 cis-element was PCR-amplified (see "Experimental Procedures"), and the product was mixed with VDE and incubated for 16 h at 37 or 25 °C. We detected no sign of cleavage of the fragment (data not shown), leading to the conclusion that VDE does not cut DNA at this position. This is a strong indication that VDE acts as a transcriptional regulatory factor for GSH11.

Effect of Rapamycin on the Expression of GSH11—It is well established that S. cerevisiae cells respond to both the quality and quantity of nutrients and that the TOR proteins, via their kinase activities, are responsible for maintaining a balance between protein synthesis and degradation in response to changes in nutritional conditions (34). For example, the TOR system up-regulates genes involved in ribosome biosynthesis, and inhibition of the expression of these genes induces autophagy. It is also known that the TOR system modulates the expression of genes responsible for the biosynthesis of various amino acids and amino acid permeases (34). Because GSH11 is induced by deprivation of sulfur, we suspected that GSH11 would be under the control of the TOR system. To test this, we examined the effect of rapamycin, an inhibitor of the TOR system (34), on the expression of GSH11. We transformed strains YPH500 (VMA1), YOC2176 (vde-delta), YOC2176/pYO314-VMA1, and YOC2176/YCpTV-VDEc with plasmid EUG11-9. The obtained transformants were incubated in SF medium in the presence or absence of 200 nM rapamycin and with or without 0.1 mM cysteine. It can be seen (Fig. 4) that in the VMA1 strain rapamycin caused an increase of β-galactosidase activity only in the absence of cysteine. Contrastingly, in the vde-delta strain rapamycin did not affect β-galactosidase regardless of the presence or absence of cysteine. This result strongly indicates that GSH11 is under the control of the TOR regulatory system and that this regulatory system is mediated by VDE. However, it appears that the expression of GSH11, unlike that of many other metabolic genes, is also coupled to another regulatory system specific for sulfur starvation and, especially, cysteine depletion (see “Discussion”).
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**DISCUSSION**

We have found that at least eight proteins bind to the 
5'-upstream region of *GSH11*. The most prominent turned out to be VDE, a protein produced by cleavage of the *VMA1* gene product Vma1p. Vma1p consists of three segments, A, B, and C, and is processed to produce two peptides, B and AC. Peptide B corresponds to VDE, and AC to the vacuolar H^+-ATPase (35). VDE acts as a homing endonuclease that cuts the VDE-free *VMA1* product at a specific site, and this triggers DNA repair using the VDE-containing *VMA1* gene on the homologous chromosome as template. As a consequence, a heterozygous diploid chromosome as template. As a consequence, a heterozygous diploid strain and the plasmids containing *VMA1* and VDE. Their information about VDE was most valuable for development of this work.

It is worth mentioning that several genes (or open reading frames) have 5'-upstream sequences identical or similar to the cis-acting motif of *GSH11*. Of these, CTS3 (coding for cystathionine γ-lyase), MET13 (coding for a putative 5,10-methenyltetrahydrofolate reductase), SPE2 (coding for S-adenosylmethionine decarboxylase), and MET2 (coding for homoserine O-acetyltransferase) are unquestionably involved in sulfur metabolism (1). Whether VDE is involved in the regulation of these genes is another interesting question, and examination of this possibility is underway in our group.

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