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Met31p and Met32p, Two Related Zinc Finger Proteins, Are Involved in Transcriptional Regulation of Yeast Sulfur Amino Acid Metabolism

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Sulfur amino acid metabolism in *Saccharomyces cerevisiae* is regulated by the level of intracellular *S*-adenosylmethionine (AdoMet). Two *cis*-acting elements have been previously identified within the 5′ upstream regions of the structural genes of the sulfur network. The first contains the CACGTG motif and is the target of the transcription activation complex Cbf1p-Met4p-Met28p. We report here the identification of two new factors, Met31p and Met32p, that recognize the second *cis*-acting element. Met31p was isolated through the use of the one-hybrid method, while Met32p was identified during the analysis of the yeast methionine transport system. Met31p and Met32p are highly related zinc finger-containing proteins. Both LexA-Met31p and LexA-Met32p fusion proteins activate the transcription of a $LexA_{op}$-containing promoter in a Met4p-dependent manner. Northern blot analyses of cells that do not express either Met31p and/or Met32p suggest that the function of the two proteins during the transcriptional regulation of the sulfur network varies from one gene to the other. While the expression of both the *MET3* and *MET14* genes was shown to strictly depend upon the presence of either Met31p or Met32p, the transcription of the *MET25* gene is constitutive in cells lacking both Met31p and Met32p. These results therefore emphasise the diversity of the mechanisms allowing regulation of the expression of the methionine biosynthetic genes.

In the yeast *Saccharomyces cerevisiae*, the sulfur amino acid biosynthesis pathway is composed of more than 20 unlinked genes whose expression is coordinately regulated: the transcription of the genes is turned off in response to an increase in the intracellular concentration of *S*-adenosylmethionine (AdoMet), the end product of this pathway (30). Owing to the numerous genetic and biochemical data accumulated so far, this metabolic pathway constitutes a model system for studying the molecular mechanisms allowing specific regulation of gene expression in eucaryotic cells.

Previous results have shown that, to a large extent, transcriptional activation of the structural genes from the sulfur network is achieved through the assembly of a multisubunit protein complex which binds to the TCACGTG core sequence, a motif found in the 5′ upstream region of these genes (24). This complex associates two basic leucine zipper factors, Met4p and Met28p, with a basic helix-loop-helix factor, Cbf1p. Recent in vitro reconstitution experiments have confirmed that the Cbf1p-Met4p-Met28p complex can form without additional factors on the TCACGTG sequence present upstream of the *MET16* gene (23). Functional analysis of each subunit has demonstrated that the Cbf1p-Met4p-Met28p complex contains only one transcription activation module, which is provided by the Met4p subunit. Met4p was indeed shown to contain a unique activation domain located in the N-terminal part of the protein and whose function is controlled by the level of intracellular AdoMet (25). In contrast, the Cbf1p and Met28p subunit functions were shown to be dedicated to DNA recognition and complex formation (23). Moreover, the Cbf1p-Met4p-Met28p complex exhibits another interesting feature, being composed of two sulfur-specific factors, Met4p and Met28p, and one multifunctional factor, Cbf1p, which is involved in both regulation of sulfur amino acid metabolism and chromosome stability (2, 7, 31). Indeed, Cbf1p binds to the CDE1 sequence, one of the three DNA elements which constitute the centromeres of *Saccharomyces cerevisiae* chromosomes (1, 16).

In addition to the three components of the Cbf1p-Met4p-Met28p complex, the AdoMet-mediated regulation of the sulfur network involves the Met30 protein, a member of the WD40 protein family (32). Met30p was shown to function by inhibiting the transcription activation function of Met4p when the level of intracellular AdoMet is high. Furthermore, as expected from the results of the functional analyses of Met4p, Met30p was shown to interact in vivo with Met4p, with this interaction requiring the Met4p inhibitory region (32).

Although the Cbf1p-Met4p-Met28p and Met30p family seems to constitute the main molecular determinant allowing the specific regulation of the sulfur network, mutational analysis of the *MET25* 5′ upstream region had identified another *cis*-acting element. The deletion of this element apparently increased the level of *MET25* transcription after the cells were grown in the presence of a repressive amount of methionine (22, 30). To determine whether this element may constitute a real regulatory component, we wanted to isolate DNA binding factors capable of recognizing this short sequence. We report in this paper the identification of two such factors. The first was identified by the one-hybrid method, and the second was isolated during a search for the genetic determinants of the methionine transport system. These two factors, Met31p and Met32p, were shown to be members of the zinc finger DNA binding factor family. Met31p and Met32p appear to act along with the Cbf1p-Met4p-Met28 complex to promote the coordinated expression of the structural genes from the sulfur amino acid biosynthesis pathway.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
CA48-6D	*MAT***a** *ade2 his3 leu2 ura3 trp1 mup1 met32*	This study
CA350-13B	*MAT*α *leu2 ura3 trp1 mup1*	20
CA350-22C	*MAT***a** *ade2 his3 leu2 ura3 trp1 mup1*	20
H461	*MAT***a** *ade2 his3 leu2 trp1 ura3 met32*::*URA3*	H. Ronne
CD106	*MAT***a** *ade2 his3 leu2 trp1 ura3 met4*::*TRP1*	24
CD158	*MAT***a** *ade2 his3 leu2 trp1 ura3 met31*::*HIS3*	This study
CD159	*MAT*α *ade2 his3 leu2 trp1 ura3 met32*::*HIS3*	This study
CD164	*MAT***a** *ade2 his3 leu2 trp1 ura3 met31*::*TRP1 met32*::*HIS3*	This study
YM954	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 gal4 gal80*	M. Johnston
YMPL1	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 gal4 gal80 URA3*::*cyc1-MET*$_{cis}$*-HIS3*	This study
X2180-1A	*MAT***a**	YGSC[a]
W303-1A	*MAT***a** *ade2 his3 leu2 trp1 ura3*	R. Rothstein
W303-1B	*MAT*α *ade2 his3 leu2 trp1 ura3*	R. Rothstein

[a] Yeast Genetic Stock Center.

MATERIALS AND METHODS

Strains, media, and microbiological techniques. The *S. cerevisiae* strains used in this work are listed in Table 1. Standard yeast media were prepared as described by Cherest and Surdin-Kerjan (8). *S. cerevisiae* was transformed after lithium acetate treatment as described by Gietz et al. (12). Genetic methods were performed as described by Sherman et al. (28).

To screen for strains unable to use low concentrations of methionine sulfoxide (MetO; 0.05 mM), strain CA350-22C (*mup1*) was mutagenized with ethyl methanesulfonate to 50% survival and mutagenesis was followed by nystatin enrichment (11). A total of 20,000 viable cells were plated (500 per petri dish) on sulfurless B medium containing all the growth requirements for strain CA350-22C and 0.5 mM L-methionine. The colonies were allowed to grow for 72 h at 28°C and were then replica plated on the same medium and on a medium containing 0.05 mM MetO instead of methionine. Of the 10 selected MetO$^-$ strains, 6 were also found to be unable to grow on homocysteine as the sulfur source and were not studied further. The four resulting mutants (MAD5, MAD10, MAD13, and MAD14) unable to grow on 0.05 mM MetO but capable of growing on homocysteine were analyzed through various crosses. The genetic analyses of these strains revealed that, in each case, the inability to grow on 0.05 mM MetO was due to one mutation in addition to the *mup1* lesion. Moreover, the MAD13 and MAD14 strains were mutated in the same gene. The three genes whose mutation led to a MetO$^-$ phenotype in the presence of a *mup1* lesion were called *MSO1*, *MSO2*, and *MSO3*.

Recombinant DNA methods. To clone the *MET31* gene, the yeast hybrid expression library used was a pool of three libraries corresponding to the insertion of a partial *Mbo*I digest of chromosomal DNA in three vectors, pGAD1F, pGAD2F, and pGAD3F (Clontech, Palo Alto, Calif.). The *S. cerevisiae* genomic library used for the cloning of *MET32* was constructed by inserting the product of a partial *Hin*dIII digest of chromosomal DNA from the wild-type strain X2180-1A into the *Hin*dIII site of pEMBLYe23. For subcloning experiments, we used the shuttle vectors pEMBLYe23 (3) and pRS316 (29). Amplifications were performed with the Pfu DNA polymerase (Stratagene, La Jolla, Calif.) and verified by sequence analysis. For the repression assays, we used the JK1261 and CK30 plasmids, which contain four *lexA* operators upstream or one *lexA* operator downstream of the *CYC1* upstream activation sequence (UAS), respectively (21). For the activation assays, we used the *GAL1-lexop-lacZ* reporter gene which has UAS_{GAL} deleted and instead contains four *lexA* operators 170 bp upstream of the *GAL1* TATA box (15).

Plasmids. The reporter gene used for the one-hybrid method was constructed by cloning the *CYC1* 5′ upstream region, with its UAS deleted, from the pLG67OZ plasmid (13) in front of the *HIS3* gene in a yeast integrative plasmid (pEMBLYi22 [3]) leading to plasmid pYi2267OHIS. This plasmid contains a unique *Xho*I site 200 bp upstream of the *CYC1* TATA box and a unique *Stu*I site within the *URA3* gene, allowing directed integration at the *URA3* chromosomal gene. To insert oriented copies of the *MET25* 5′ *cis*-acting element in this plasmid, we used the following oligonucleotide duplex:

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5′ ATCCTCATGAAAACTGTGGAACA 3′
     3′ GTACTTTTGACACCTTGTTAGGA 3′
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After its phosphorylation by T4 DNA kinase, the oligonucleotide duplex was autoligated overnight at 14°C, treated with Klenow fragment to blunt the ends, and ligated with the pYi2267OHIS plasmid which was previously cleaved with *Xho*I, blunt ended by the Klenow fragment, and dephosphorylated. The orientation and the copy number of inserted elements were determined by sequence analysis. The resulting plasmid was then cleaved with *Stu*I and used to transform the YM954 strain. Stable uracil prototroph transformants were selected, and correct integration events were verified by Southern blot analysis. The resulting YMPL1 strain used for the cloning of the *MET31* gene contained two copies of the *MET25* 5′ *cis*-acting element.

To construct the derivatives of Met31p and Met32p, each corresponding open reading frame (ORF) was amplified by PCR with specific oligonucleotides. Plasmids expressing derivatives of either Met31p or Met32p fused to the activation domain of Gal4p were derived from plasmid pGAD424 (4). Plasmids expressing derivatives of either Met31p or Met32p fused to the DNA binding domain of LexA were derived from plasmid pEG202 (14). For the expression of histidine-tagged derivatives of Met31p and Met32p in *Escherichia coli*, plasmids pETMet31 and pETMet32 were derived from the pET28a vector (Novagen, Madison, Wis.). To inactivate the chromosomal copies of either *MET31* or *MET32*, the corresponding genes were first cloned into plasmid pUC9EO (30). For *MET31*, a selectable marker, *HIS3* or *TRP1*, was inserted at the unique *Eco*RI site within the *MET31* ORF while for *MET32*, the selectable marker *HIS3* was inserted at the unique *Sma*I site within the *MET32* ORF. The resulting plasmids were linearized by *Bam*HI and *Sph*I and by *Bam*HI and *Eco*RI, respectively, and used to inactivate the corresponding chromosomal loci by the one-step gene disruption method (27). Correct inactivations were confirmed by PCR amplifications.

Protein purifications. Bacterially synthesized histidine-tagged recombinant Met31 and Met32 proteins were purified by affinity chromatography on nickel-agarose columns. *E. coli* BL21(λDE3) cells harboring either pETMet31 or the pETMet32 were grown at 37°C in 500 ml of Luria broth containing 50 μg kanamycin per ml to an optical density at 650 nm of 1. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the growth was continued for another 3 h at 37°C. The following procedures were performed at 4°C with ice-cold buffers. Cells were harvested by centrifugation, resuspended in 1 volume of buffer A (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 5 mM imidazole, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, and lysed by passage of the suspension through an Eaton press. The cell extract was diluted with 1 volume of buffer A containing 1 mM phenylmethylsulfonyl fluoride, 10 mg of RNase A per ml, and 5 mg of DNase I per ml and incubated for 15 min in ice. Insoluble material was removed by centrifugation at 30,000 × *g* for 30 min. The clear lysate was then loaded onto a 4-ml Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen, Hilden, Germany), and chromatography was performed at a flow rate of 0.5 ml/min. The loaded column was washed with 40 ml of buffer A and then with 120 ml of buffer B (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 60 mM imidazole, 10% glycerol). Bound proteins were eluted with buffer C (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 340 mM imidazole, 10% glycerol). The fractions containing 6HMet31 and 6HMet32 proteins were pooled and stored at −20°C. Protein concentrations were determined by the Bradford assay.

Electrophoretic mobility shift assays. All mobility shift assays were performed with 20 μl of HDB buffer (25 mM HEPES [pH 7.6], 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$), containing 0.5 mg of bovine serum albumin (BSA) per ml, 7.5% glycerol, and 0.8 μg of poly(dI-dC)-poly(dI-dC) competitor DNA with various amounts of recombinant proteins as indicated in the figure legends. Oligonucleotide probes were 5′-end labelled with T4 DNA kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham). Approximately 20,000 cpm of probe (about 0.25 ng) was used in each binding mixture. The samples were incubated for 15 min in ice before being loaded onto an 8% polyacrylamide gel in 0.25× TBE (22 mM Tris [pH 8.3], 22 mM boric acid, 0.6 mM EDTA) and electrophoresed at 9 V/cm at 7°C. The gels were preelectrophoresed for 45 min at 5 V/cm at 7°C. The gels were run for 5 h, dried, and subjected to autoradiography for 15 h with an intensifying screen.

DNase I footprinting assays. Probes for DNase I footprinting were prepared by PCR amplification, except that only a single primer was labelled with [γ-^{32}P]ATP. The probes (80,000 cpm) were incubated with the indicated proteins in 20 μl of HDB buffer containing 0.5 mg of BSA per ml, 7.5% glycerol, and 0.4 μg of poly(dI-dC)-poly(dI-dC) on ice for 15 min. DNase I (Sigma) was diluted in 10 mM HEPES (pH 7.6)–25 mM $CaCl_2$–100 μg of BSA per ml to 2.5 ng/μl. Then 2 μl was added to the binding-reaction mixture, and digestion was continued for 5 min on ice. The reaction was stopped by addition of 200 μl of stop buffer (2.5 M ammonium acetate, 50 μg of tRNA per ml). The DNA was ethanol precipitated, dried, and resuspended in 12 μl of formamide dye. A 3-μl volume was loaded on an 7% acrylamide–7 M urea sequencing gel.

Northern blot analyses. Northern blotting was performed as described by Thomas (33), with total cellular RNA extracted from yeast as described by Hoffman and Winston (19) and oligonucleotide-labelled probes (18).

Methionine uptake assays. Assays for methionine uptake were performed as described previously (20). Briefly, the assays were done with exponential-phase cells in minimal medium containing ammonia as the sole nitrogen source. Labelled methionine (about 2000 cpm/nmol) was added to a final concentration of 0.02 mM unless otherwise stated. Samples were taken each 1 min for 4 min and filtered through fiberglass filters. Each filter was washed with 10 ml of cold distilled water and counted in a scintillation counter. For each uptake determination, we verified that uptake was linear for the 4 min. For kinetic analyses, the concentration range of methionine was 0.4 to 20 μM for wild-type cells and 0.02 to 1.6 mM for *mup1* mutated cells. The K_T values refer to the apparent K_m calculated from double-reciprocal plots (20).

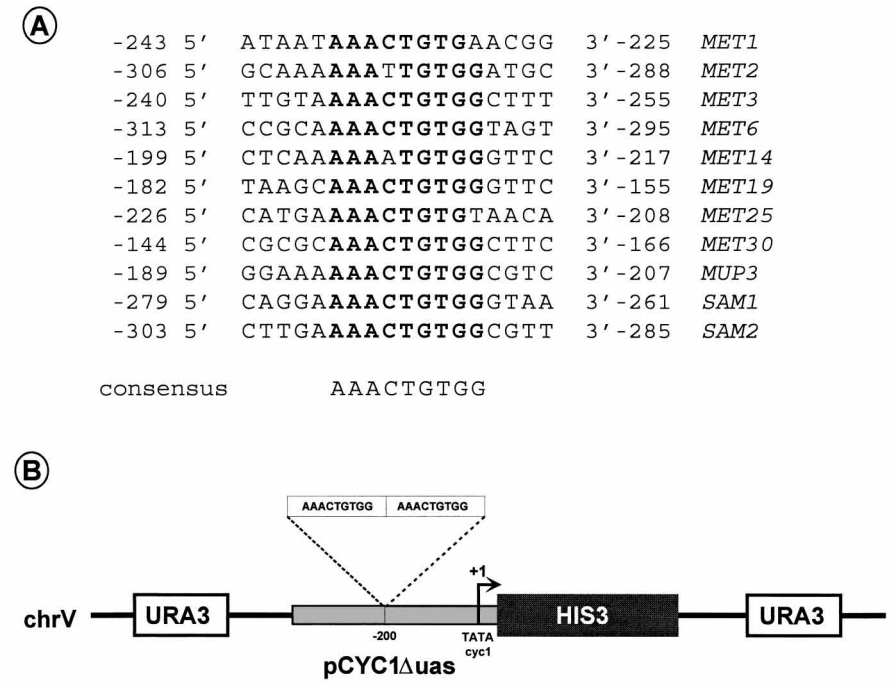

FIG. 1. (A) Alignment of the conserved sequence found in the 5′ upstream regions of the structural genes governing the sulfur amino acid metabolism. (B) Structure of the *HIS3* reporter gene construct used for the one-hybrid experiment.

RESULTS

As previously reported, a mutational analysis of the 5′ upstream region of the *MET25* gene had identified two regions important for the expression of this gene (30). The first contained a repetition of the CACGTG sequence which is now known to be recognized by the Cbf1p-Met4p-Met28p complex (24). The deletion of this region severely impaired the expression of *MET25*. The second region was localized around nucleotide −200 (numbered relative to the initiation codon). Deletions of this region resulted mainly in an increase of *MET25* gene expression under repressive growth conditions. However, weak decreases of the transcription of *MET25* under nonrepressive growth conditions were also noted (30). Although the results of these experiments did not precisely reveal the role of the −200 region, its functional implication in transcription regulation was suggested by the fact that it contains a short sequence which was also found upstream of several methionine biosynthetic genes (20, 22, 30). Indeed, as shown in Fig. 1, a careful examination of the 5′ upstream regions of the coregulated genes revealed that most contained the consensus sequence 5′ AAACTGTGG 3′. To address the functional significance of this sequence, we decided to use the one-hybrid method to search for yeast proteins that specifically recognize this sequence.

Identification of the *MET31* gene by the one-hybrid method. To identify yeast genes encoding factors that could recognize the 5′ AAACTGTGG 3′ sequence, we constructed a reporter gene consisting of two tandem copies of a 26-bp oligonucleotide containing the −200 element and its adjacent nucleotides from the *MET25* promoter region modified to match the consensus sequence. The oligonucleotides were inserted 200 bp upstream of the TATA box of the *CYC1* promoter, with its own UAS deleted, and placed in front of the *HIS3* gene. This construct was integrated into the genome of a *his3 gal4 gal80* mutant strain (YM954) at the *URA3* locus. The resulting strain (YMPL1) exhibited a leaky His^+ phenotype, which was suppressed by the addition of 25 mM aminotriazole (AT) to the medium. The YMPL1 strain was transformed by a yeast hybrid expression library which contained random protein segments fused to the Gal4p transcriptional activation domain (10). Transformants were selected directly for growth in the absence of histidine and the presence of 25 mM AT. From a screen of about 5.2×10^5 transformants, 45 colonies able to grow in the presence of 25 mM AT appeared over the course of 10 days. Plasmid DNA was recovered from these colonies and used to retransform the YMPL1 strain. Only one plasmid (pBL12) gave rise to transformants that were able to grow in the presence of 25 mM AT. The junction between the Gal4p activation domain and the genomic DNA present on plasmid pBL12 was sequenced and revealed an in-frame fusion between the Gal4p activation domain and the last 70 amino acids of the YPL039w ORF identified on the left arm of chromosome XVI by the systematic sequencing of the yeast genome. The YPL039w ORF is predicted to code for a protein of 177 amino acid residues which contains two zinc finger motifs, suggesting that it may be a transcription factor. It must be noted that the Gal4-YPL039w derivative encoded by the isolated plasmid contained only the carboxy-terminal zinc finger, which therefore appears to be responsible for the recognition of the *cis* element. The YPL039w ORF was thus called *MET31.*

Isolation of Met32p mutants and cloning of the corresponding gene. The *met32* mutation was isolated during the analysis of methionine transport in *S. cerevisiae*. We recently demonstrated that yeast cells contain three different methionine permeases, which are encoded by the *MUP1*, *MUP2*, and *MUP3* genes (20). The *MUP1* and *MUP3* genes encode highly related products corresponding to the high- and very-low-affinity methionine permeases, respectively. In an attempt to identify the *MUP2* gene, we searched for mutations that, in the presence of a mutation in *mup1*, would lead to cells unable to grow on a

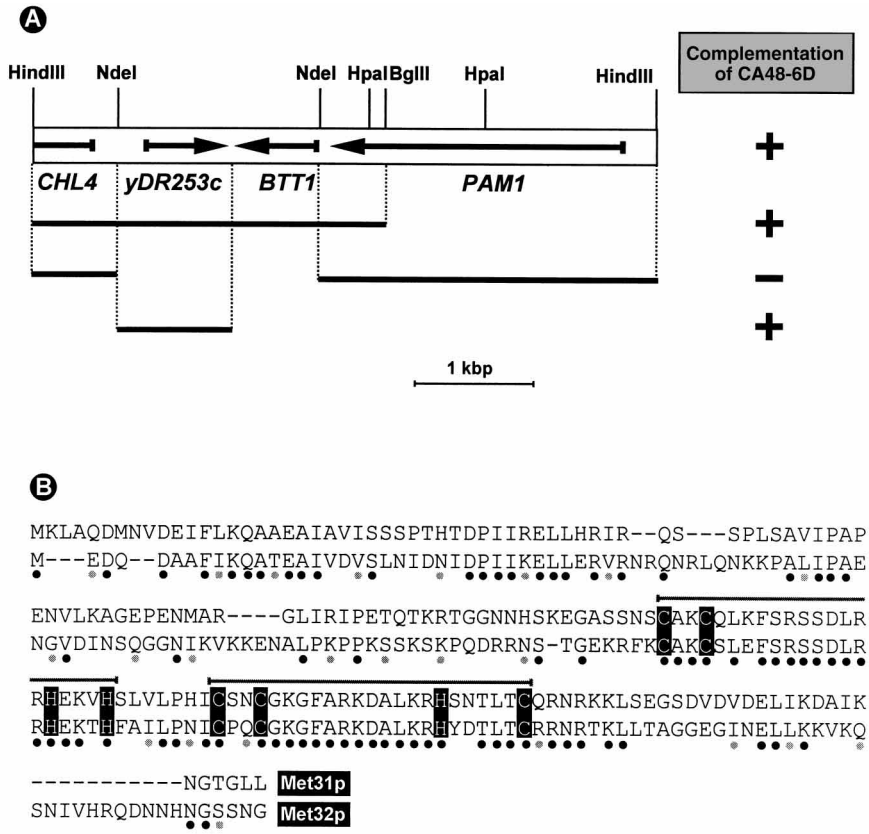

FIG. 2. (A) Physical map of the YDR253c (*MET32*) region. The fragments subcloned in plasmid pEMBLYe23 and their ability to confer to strain CA48-6D (*mup1 met32*) the capacity to grow on 0.05 mM MetO are shown. (B) Alignment of Met31p and Met32p. The two proteins were aligned by using the Clustal V program (17). Solid circles indicate identities. Grey circles indicates conservative replacements. Grey lines indicate the two zinc finger regions (the histidine and cysteine residues are enclosed in black boxes).

low concentration (0.05 mM) of MetO used as the sulfur source. As described in Materials and Methods, this specific genetic screen allowed us to isolate three mutations that identify three unlinked new genes, provisionally called *MSO1*, *MSO2*, and *MSO3*. However, it must be noted that even in the presence of the two mutations *mup1* and *mso1*, a residual growth on MetO was observed. First, enzymatic analysis of the double *mup1 mso1* mutant strain confirmed that the *mso1* mutation affects the methionine transport system (data not shown [see below]). We next tried to clone the genomic DNA corresponding to the *MSO1* locus. Strain CA48-6D (*mup1 mso1 ura3*) was thus transformed by a pEMBLYe23-based genomic library, and 10^5 transformants able to grow in the absence of uracil were selected on 20 plates. These transformants were recovered by washing the plates, the resulting cell suspensions were plated onto a synthetic sulfurless B medium containing 0.05 mM L-MetO, and fast-growing cells were selected. To determine whether the fast-growing phenotype was due to plasmid information, 100 clones were replicated twice on complete medium containing 5-fluoroorotic acid to induce plasmid loss (6) and then tested again on B medium containing 0.05 mM L-MetO. This allowed us to identify 12 clones whose $MetO^+$ phenotype indeed resulted from the presence of a plasmid. Plasmid DNA was then isolated from the 12 colonies and shown to correspond to two groups. The first one comprised plasmids containing an 8.7-kbp insert, and the second comprised plasmids containing a 5.2-kbp insert. When these plasmids were used to retransform the CA48-6D strain, all the uracil prototroph transformants were shown to exhibit fast growth on 0.05 mM L-MetO. The restriction map of the 8.7-kbp insert revealed that it contained the *MUP1* gene (20). The sequence of the 5.2-kbp insert extremities revealed that it encompassed the *PAM1-CHL4* genomic region of the right arm of chromosome IV. This region also contains the *BTT1* gene and one ORF (YDR253c) of unknown function. Subcloning experiments showed that a small insert containing only the YDR253c ORF was sufficient to complement the $MetO^-$ phenotype of strain CA48-6D (Fig. 2A). To determine whether the insert directed integration to the *MSO1* region of the yeast genome, strain CC350-13B (*ura3 mup1 MSO1*) was transformed with a plasmid lacking an autonomous yeast replicating sequence but bearing the *URA3* gene and the complementing insert. Integration was directed by cleaving the plasmid within the insert with *Bgl*II. One Ura^+ transformant was crossed with strain CA48-6D, the diploid was sporulated, and tetrads were dissected. In all cases (20 tetrads), the $MetO^+$ phenotype segregated 2+/2− and all of the $MetO^+$ segregants were uracil prototrophs. These results indicate that the insert directed integration to the *MSO1* locus and therefore that the *MSO1* gene corresponds to the YDR253c ORF.

Sequence analysis of YDR253c revealed that it codes for a small protein of 191 amino acids that contains two zinc finger motifs. Moreover, as shown in Fig. 2B, the YDR253c-encoded protein exhibit strong similarity to Met31p. Alignments obtained with the Clustal V program show that the two proteins have 81 identical residues (46%) and 23 conservative replacements (13%). We thus decided to call *MET32* the *MSO1* (YDR253c) gene.

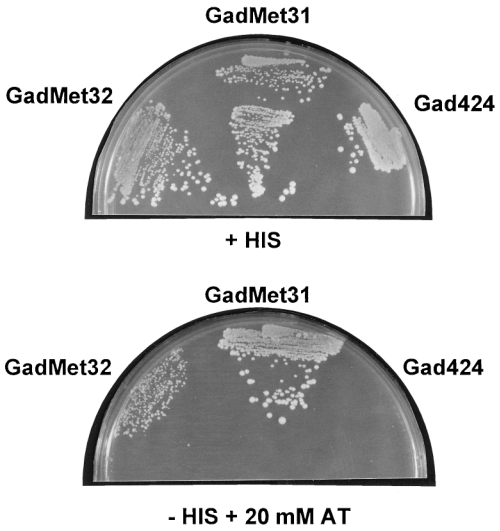

FIG. 3. AT resistance of YMPL1 cells expressing the GadMet31 or GadMet32 fusion protein. YMPL1 cells (*URA3*::*cyc1-MET*$_{cis}$*-HIS3*) were transformed either by plasmids expressing the GadMet31 or the GadMet32 fusion proteins or by the parental vector pGAD424. Transformants were streaked on a histidine-containing medium or on a medium lacking histidine but containing 20 mM AT.

Met31p and Met32p recognize the 5′ AAACTGTGG 3′ sequence. To determine whether Met31p and Met32p are indeed capable of recognizing the 5′ AAACTGTGG 3′ sequence, we constructed fusions between the Gal4 activation domain and either the complete Met31p or the complete Met32p. We then tested whether YMPL1 cells expressing each of the fusion proteins were able to grow in presence of 25 mM AT. As shown in Fig. 3, expression of either GadMet31 or GadMet32 fusion protein rendered the cells AT resistant. It must be noted, however, that cells expressing the GadMet31 fusion protein appeared to be more resistant to 25 mM AT than were those expressing the GadMet32 fusion protein (Fig. 3).

Next, Met31p and Met32p were expressed in *E. coli* as histidine-tagged proteins and subsequently purified by affinity chromatography on agarose-nickel columns (see Materials and Methods). The recombinant proteins were assayed in mobility shift assays with a fragment of the *MET25* promoter encompassing nucleotides −270 to −170 and containing the 5′ AAA CTGTG 3′ sequence. As shown in Fig. 4, the two recombinant 6HMet31 and 6HMet32 proteins are capable of binding to the *MET25* probe and addition of unlabelled oligonucleotides containing the 5′ AAACTGTGG 3′ sequence to the DNA binding reaction mixtures decreased the amount of the formed complexes, confirming that both Met31p and Met32p recognize this upstream element. When the 6HMet31 protein was added to the DNA binding reaction mixtures, several complexes with slow mobility and low intensity are also observed. It is possible that they resulted from low-affinity binding of the 6HMet31 protein to different sites; alternatively, these complexes may arise from the multimerization of Met31p on DNA, as previously observed for several DNA binding factors (9).

To confirm all these results, DNase I protection experiments were performed. As shown in Fig. 5, the presence of either the 6HMet31 or 6HMet32 recombinant protein in the DNA binding reaction mixtures led to a footprint over the 5′ AAACTG TGG 3′ sequence, confirming that the two proteins indeed recognize this upstream element.

Transcription activation by either LexAMet31 or LexAMet32 requires Met4p. Since the 5′ AAACTGTGG 3′ sequence was

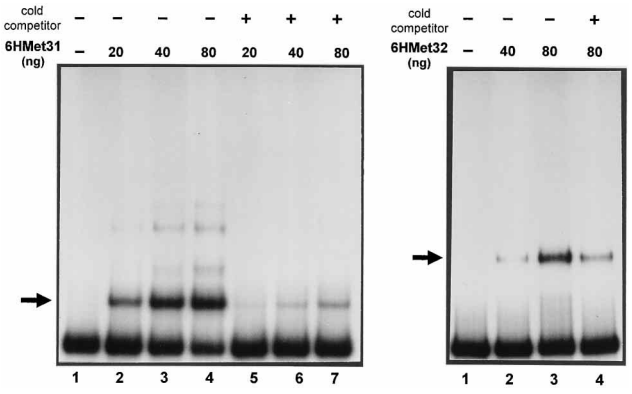

FIG. 4. Recombinant 6HMet31 and 6HMet32 proteins bind to the *MET25* 5′ upstream region. The indicated amounts of either 6HMet31 or 6HMet32 recombinant proteins were incubated with a *MET25* probe corresponding to nucleotides −270 to −170 in the absence or presence of a 100-fold excess of the unlabelled oligonucleotide containing the 5′ AAACTGTGG 3′ sequence.

first identified as a *cis*-acting regulatory element potentially required for complete repression of the *MET25* gene transcription, we began the functional analysis of Met31p and Met32p by asking whether they may function as repressors. We tested whether Met31p and Met32 could repress the actvity of a promoter when brought to DNA. We constructed protein fusions between the bacterial LexA protein and either the complete Met31p or the complete Met32p and tested these con-

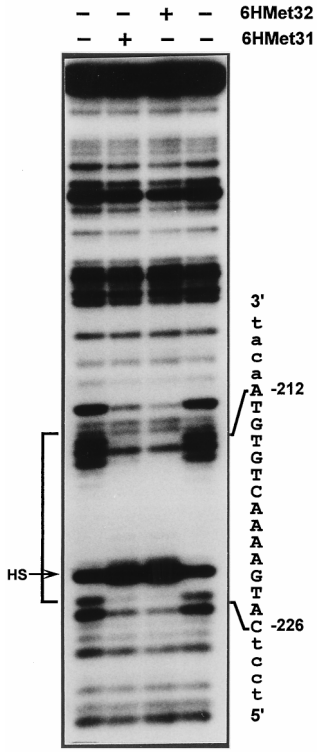

FIG. 5. DNase I footprint assays of 6HMet31 and 6HMet32 proteins on the *MET25* 5′ upstream region. The panel show the upper strand of the *MET25* 5′ upstream region. Binding reactions were performed with 80 ng of either 6HMet31 or 6HMet32 recombinant protein. The solid bar marks the footprint over the 5′ AAACTGTGG 3′ sequence, and HS indicated the major hypersensitive site.

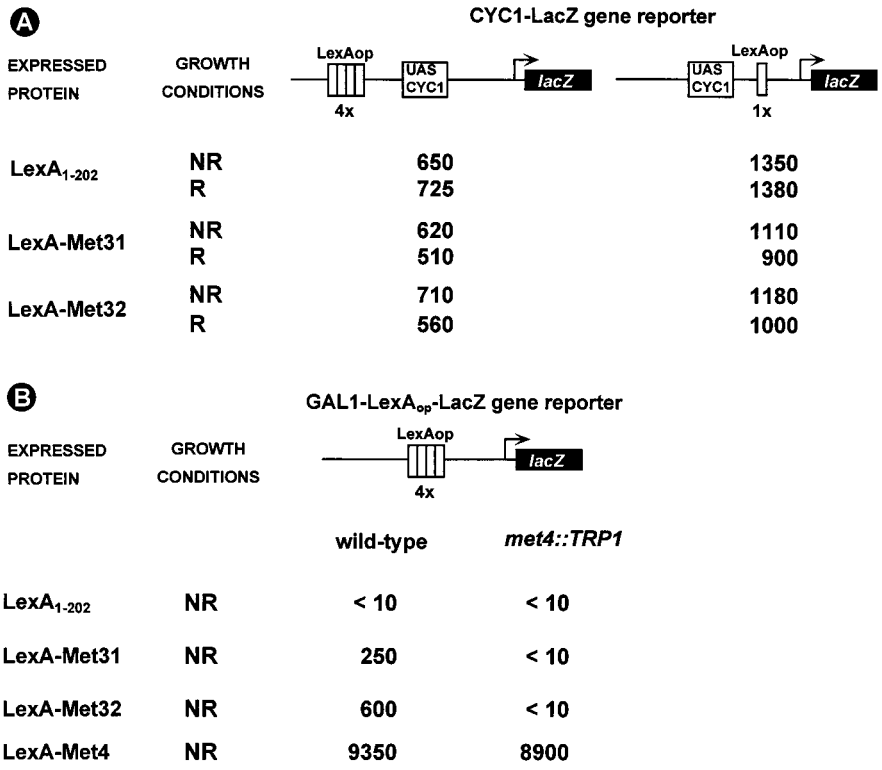

FIG. 6. The LexAMet31 and LexAMet32 proteins function as transcription activators. (A) Repression assays. YM954 cells were cotransformed by either the JK1261 (four *lexA* operators) or the CK30 (one *lexA* operator) reporter construct and a plasmid expressing either the LexA-bicoid, the LexAMet31, or the LexAMet32 fusion proteins. (B) Activation assays. W303-1A and CD106 (*met4::TRP1*) cells were cotransformed by the pSH18-34 (four *lexA* operators in place of the $GAL1_{UAS}$) reporter construct and plasmid expressing either the $LexA_{1-202}$, the LexAMet31, or the LexAMet32 fusion protein. β-Galactosidase activities are expressed in nanomoles per minute per milligram of protein. NR, nonrepressive growth conditions; R, repressive growth conditions (1 mM L-Met).

structions using *CYC1-lacZ* reporter genes that contain either four *lexA* operators upstream or one *lexA* operator downstream of the *CYC1* UAS (plasmids JK1261 and CK30, respectively [21]). The results of the β-galactosidase assays performed with the cotransformed cells (Fig. 6A) grown under either nonrepressive or repressive growth conditions demonstrated that neither the expression of the LexAMet31 protein nor that of the LexAMet32 protein affected the activity of the reporter genes, therefore suggesting that Met31p and Met32p do not function as repressors.

To examine further the role of Met31p and Met32p, we tested whether the LexAMet31 and LexAMet32 fusion proteins were capable of activating the transcription of a *GAL1-lacZ* reporter gene with four *lexA* operators replacing the UAS (15). The β-galactosidase assays thus gave a measure of the transcription activation function of each protein since the DNA binding was achieved through the LexA moiety. As shown in Fig. 6B, both the LexAMet31 and LexAMet32 fusion proteins activated the transcription of the reporter gene when they were expressed in wild-type cells. Therefore, both Met31p and Met32p seemed to function as transcription activators. However, the β-galactosidase activity resulting from the expression of either LexAMet31 or LexAMet32 was lower than that measured with cells expressing the LexAMet4 fusion protein. To test whether the activity of the LexAMet31 and LexAMet32 fusion proteins could require the Met4 function, we expressed the two fusion proteins in cells containing a disruption mutation at the *met4* locus. As shown in Fig. 6B, the disruption mutation of *met4* virtually abolished the activation of the *lacZ* reporter gene by the LexAMet31 and LexAMet32 fusion proteins. Thus, both Met31p and Met32p appear to be devoid of intrinsic transcription activation function.

Role of Met31p and Met32p in the transcription activation of the sulfur amino acid metabolism. To determine the role of Met31p and Met32p in the regulation of sulfur amino acid metabolism and to find whether the two proteins may have different functional targets, we constructed strains that do not express either Met31p or Met32p. This was done by inserting selectable markers within the corresponding genes (see Materials and Methods) by the one-step gene disruption method in either haploid or diploid strains. Both *met31*- and *met32*-disrupted haploid strains were viable and did not exhibit severe phenotypic alterations. In contrast, the *met31 met32* doubly disrupted strain (CD164) was a methionine auxotroph (Fig. 7). Actually, the CD164 cells can grow only in the presence of methionine or AdoMet. Homocysteine or inorganic sulfur does not support the growth of strain CD164. Moreover, in the presence of methionine or AdoMet, the CD164 cells grew more slowly than wild-type cells and reached the stationary phase at a lower cell density (Fig. 7). This result may arise from methionine or AdoMet uptake impairment or may suggest that the functions of Met31p and Met32p are not restricted to the sulfur amino acid metabolism.

Since *met32* mutations were isolated as lesions affecting the methionine transport system, we compared the kinetics of methionine uptake in cells lacking either Met31p or Met32p to that measured in their parental strain. This was done with a broad range of methionine concentrations as described previously (20). As shown in Table 2, methionine uptake assays revealed that the CD158 cells (*met31*::*HIS3*) lack the very-low-

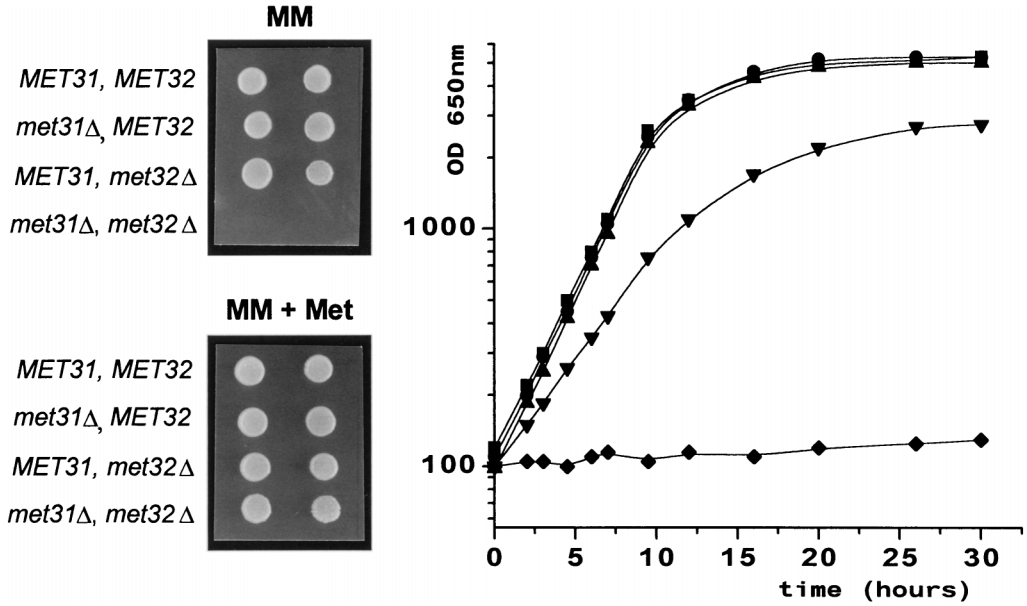

FIG. 7. The *met31 met32* doubly disrupted strain is a methionine auxotroph. The CD158 (*met31*::*HIS3*), CD159 (*met31*::*HIS3*), and CD164 (*met31*::*TRP1 met32*::*HIS3*) strains and their parental wild-type strain, W303-1A, were tested for growth in the absence or presence of methionine. Symbols: ●, W303-1A strain grown in minimal medium; ■, CD158 strain grown in minimal medium; ▲, CD159 strain grown in minimal medium; ◆, CD164 strain grown in minimal medium with 0.2 mM DL-homocysteine; ▼, CD164 strain grown in minimal medium with 0.1 mM L-methionine. OD, optical density; MM, minimal medium.

affinity methionine permease (K_T = 1 mM; encoded by the *MUP3* gene) while no methionine uptake corresponding to the low-affinity permease (K_T = 0.2 mM; encoded by the *MUP2* gene) could be detected in CD159 cells (*met32*::*HIS3*).

The role of Met31p and Met32p was further studied by comparing the transcription of several genes from the sulfate assimilation pathway in cells that do not express either Met31p or Met32p. We measured the transcription of the *MET3*, *MET14*, and *MET25* genes, each containing the AAACTG TGG sequence in their 5′ upstream region (Fig. 1). The Northern experiments were performed by shifting the cells from a medium containing a repressing amount of methionine to one without methionine and extracting the RNAs at regular time intervals after the shift. As reported previously (26) in wild-type cells the derepression kinetics are the same for the three genes, whose transcription reaches its maximal level 80 min after the shift. Unexpectedly, the single *met31* and *met32* disruption mutations led to opposite regulatory effects. In *met31*::*HIS3* cells, the mRNAs of the three genes appear more rapidly than in wild-type cells, being detected first 20 min after the shift (Fig. 8). In contrast, the *met32*::*HIS3* disruption mutation affects the expression of the *MET3*, *MET14*, and *MET25* genes by increasing the time needed for the transcription of these genes to reach its maximal level (Fig. 8). The results therefore suggest that Met31p and Met32p may play antagonist functions during the transcriptional regulation of the methionine biosynthetic genes. However, this interpretation is complicated by the analysis of the *met31 met32* doubly disrupted mutant cells. In such cells, *MET3* and *MET14* transcripts could not be detected whereas *MET25* transcription was activated, albeit at a lower level than in wild-type cells (Fig. 8). Moreover, in the absence of both Met31p and Met32p, transcription of the *MET25* gene was rather constitutive, being only weakly changed during the shift from a medium with methionine to one without methionine. This last result explains why the 5′ AAACTGTGG 3′ sequence has been first described as a repressing *cis*-acting element during the mutational analysis of the 5′ upstream region of the *MET25* gene (30). Taken together, all these results show that the function of the 5′ AAACTGTGG 3′ sequence varies from one gene to another.

DISCUSSION

The sulfur amino acid metabolism in yeast constitutes a regulatory network whose genes are regulated mainly in response to one cellular signal: the increase in the amount of intracellular AdoMet (which can be considered the ultimate product of the pathway). In spite of this obvious simplicity, genetic studies have revealed an unexpected complexity of the molecular mechanisms sustaining this regulation.

At the outset of this study, four transcriptional factors were known to be involved in this mechanism: the three subunits of the Cbf1p-Met4p-Met28p complex and the transcription inhibitor Met30p, a WD40 protein (24, 32). We report here the identification of two new factors, Met31p and Met32p, which are components of this regulatory network. Met31p and Met32p were isolated by two different experimental approaches. Met31p

TABLE 2. Methionine uptake in *met31*- and *met32*-disrupted strains

Strain	Relevant genotype	High-affinity permease		Low-affinity permease		Very-low-affinity permease	
		K_T[a]	J_{max}	K_T	J_{max}	K_T	J_{max}
W303-1A	*MET31 MET32*	0.012	14	0.11	40	1.1	58
CD158	*met31*Δ *MET32*	0.016	14	0.14	33	—[b]	—
CD159	*MET31 met32*Δ	0.010	17	—	—	1.1	50

[a] K_T and J_{max} refer respectively to the apparent K_m and V_{max} estimated from double-reciprocal plots (see Materials and Methods). For estimation of the K_T, the concentration range of methionine was 0.4 to 20 μM for the high-affinity permease and 0.02 to 1.6 mM for the low- and very-low-affinity permeases. Values of K_T are expressed in millimolar; values of J_{max} are expressed in nanomoles per minute per milligram (dry weight).

[b] —, not detectable.

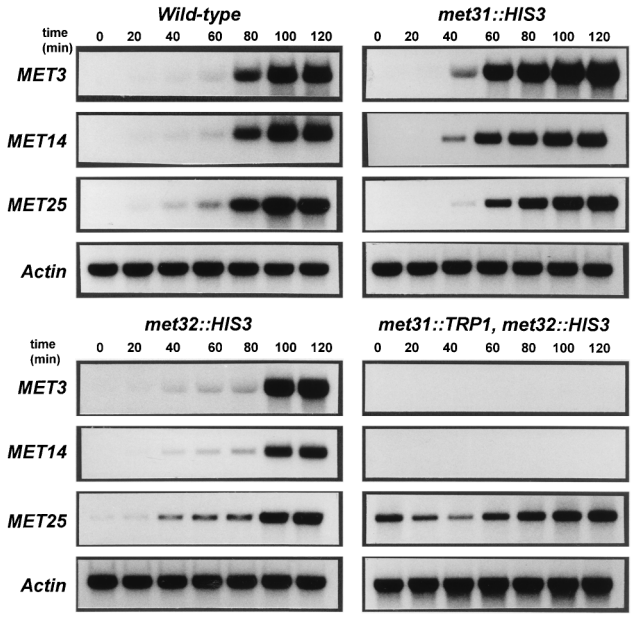

FIG. 8. Effects of *met31* and *met32* disruption mutations on the transcription of methionine biosynthetic genes. The derepression kinetics of the transcription of the *MET3*, *MET14*, and *MET25* genes were monitored in W303-1A (wild type), CD158 (*met31*::*HIS3*), CD159 (*met32*::*HIS3*), and CD164 (*met31*::*TRP1 met32*::*HIS3*). The cells were grown in 100 ml of B medium in the presence of a repressing amount of L-methionine (1 mM) as the sulfur source. When the cells reached a density of about 10^7 cells per ml, they were harvested by filtration and washed with 100 ml of B medium. They were then suspended in 100 ml of B medium without methionine and shaken at 28°C. The samples were then withdrawn at different times, and total RNAs were extracted. For each time point, 10 μg of total RNA was electrophoresed on a 1% agarose gel and transferred to a nylon membrane. The transferred RNAs were hybridized to the following radioactive fragments: the *Xho*I-*Bgl*II fragment from the *MET3* gene, the *Hpa*I-*Sca*I fragment from the *MET14* gene, and the *Xba*I-*Eco*RI fragment from the *MET25* gene (24). The actin probe was used as a control for the amounts of RNA loaded. The films were exposed for 3 days at −80°C.

was isolated by using the one-hybrid technique and searching for yeast factors capable of recognizing a *cis* element found upstream of several *MET* genes. Met32p was identified through a specific genetic screen during the analysis of methionine uptake in yeast. The cloning of the two corresponding genes revealed that they code for highly related proteins which belong to the zinc finger factor family. For each protein, the first zinc finger is of the CC/HH type while the carboxy-terminal zinc finger is of the CC/HC type, a less frequent class of zinc finger which was essentially found in retrovirus nucleic acid binding proteins (5, 34). As shown in Fig. 2B, the sequence of each zinc finger region of Met31p is very close to that of its counterpart found within Met32p, suggesting that they could share some DNA binding specificity. Accordingly, we show here that the two purified recombinant 6HMet31 and 6HMet32 proteins are both capable of recognizing the *MET25* promoter region. DNase I footprint experiments confirm that Met31p and Met32p indeed bind to the 5′ AAACTGTGG 3′ DNA sequence. Thus, from the in vitro experiments, it seemed that Met31p and Met32p form a pair of homologous DNA binding proteins.

The regulatory function of Met31p and Met32p was assessed in vivo by the analysis of the activity of LexAMet31 and LexAMet32 fusion proteins as well as by the study of cells that do not express Met31p or Met32p or both. As stated above, it was inferred from the mutational analysis of the *MET25* 5′ upstream region that the 5′ AAACTGTGG 3′ sequence might be the target of a repressor whose binding could be required to maintain to a very low level the transcription of the coregulated genes when the intracellular AdoMet concentration was high (30). However, the results presented here suggest that the function of the 5′ AAACTGTGG 3′ sequence, and therefore that of Met31p and Met32p, varies from one gene to another. First, the functional analysis of LexAMet31 and LexAMet32 fusion proteins reported here does not support the possibility that Met31p and Met32p may function by themselves as repressors. Indeed, even in cells growing under repressive growth conditions, the binding of either Met31p or Met32p in the vicinity of a functional UAS does not affect the expression of the downstream reporter gene. In contrast, we show that when expressed in wild-type cells, both LexAMet31 and LexAMet32 fusion proteins are capable of activating the transcription of a reporter gene placed downstream LexA operators. However, this transcription activation does not occur in *met4*-disrupted cells. These results suggest that Met31p and Met32p themselves are devoid of intrinsic transcription activation function. Perhaps LexAMet31 and LexAMet32 recruit Met4p, which provides the transcription activation function. Alternatively, LexAMet31 and LexAMet32 could function on *lexA* operators by recruiting another transcriptional activator whose expression depends upon Met4p.

The functional versatility of Met31p and Met32p was further revealed by analysis of the expression of several of the genes containing the 5′ AAACTGTGG 3′ sequence. Northern shift experiments revealed that in the absence of both Met31p and Met32p, neither *MET3* nor *MET14* is expressed whereas the *MET25* gene transcription appears to occur in a constitutive manner, albeit at a lower level than in wild-type cells. Therefore, Met31p and Met32p seem to function as negative *trans*-regulatory factors at the *MET25* promoter region whereas they act as essential positive effectors at the *MET3* and *MET14* promoter regions. The former result is in accord with the mutational analysis of the 5′ upstream region of the *MET25* gene: removal of the 5′ AAACTGTGG 3′ sequence found within this region results in a less repressible allele (30). It can be assumed that the different changes observed from one structural gene to the other in cells lacking both Met31p and Met32p may occur because the interactions of these two proteins with adjacent bound factors may differ on the different promoters. That the molecular mechanisms leading to the coregulation of the methionine biosynthetic genes might be diverse and might differ depending on the gene has already been postulated during the analysis of cells that do not express either Cbf1p or Met28p (24, 26). It is indeed known that *MET3* and *MET14* regulation, on one hand, and *MET25* regulation, on the other hand, are not affected in an equivalent manner by the *cbf1* and *met28* disruption mutations. Since the *met4* disruption mutation is the only known lesion that virtually abolishes the transcription activation of all the structural genes from the sulfate assimilation pathway, it is tempting to postulate that the transcriptional variations from one structural gene to the other in the different mutants in Northern shift experiments reflect the different ways by which all these factors (Cbf1p, Met28, Met31p, and Met32p) cooperate to target Met4p on the different promoter regions. Accordingly, Cbf1p, Met28p, Met31p, and Met32p were all demonstrated to be devoid of intrinsic transcription activation capacities (24, 31; see above), and Met4p is the only factor involved in the regulation of the sulfur amino acid metabolism that is known to contain a transcription activation domain. Experiments are under way to analyze how the different proteins interact on different promoters.

In contrast to what has been determined for Cbf1p, Met4p,

and Met28p, the functions of Met31p and Met32p are not restricted to the sulfate assimilation pathway. Indeed, Met31p and Met32p are both involved in regulation of the expression of several structural genes from the organic sulfur metabolism pathways like those encoding the methionine permeases (see above) as well as those encoding the two AdoMet synthases (data not shown).

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REFERENCES

1. **Baker, R. E., M. Fitzgerald-Hayes, and T. O'Brien.** 1989. Purification of the yeast centromere binding protein CP1 and a mutational analysis of its binding site. J. Biol. Chem. **264:**10843–10850.
2. **Baker, R. E., and D. C. Masison.** 1990. Isolation of the gene encoding the *Saccharomyces cerevisiae* centromere-binding protein CP1. Mol. Cell. Biol. **10:**2458–2467.
3. **Baldari, C., and G. Cesarini.** 1985. Plasmids pEMBL: new single stranded shuttle vectors for the recovery and analysis of yeast DNA sequences. Gene **35:**27–32.
4. **Bartel, P. L., C. T. Chien, R. Sternglanz, and S. Fields.** 1993. Using the two-hybrid system to detect protein-protein interactions. Oxford University Press, Oxford, United Kingdom.
5. **Berg, J. M.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science **232:**485–487.
6. **Boeke, J. D., F. Lacroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5′-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–346.
7. **Cai, M., and R. W. Davis.** 1990. Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell **61:**437–446.
8. **Cherest, H., and Y. Surdin-Kerjan.** 1992. Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. Genetics **130:**51–58.
9. **Ferré d'Amaré, A. R., P. Pognonec, R. G. Roeder, and S. K. Burley.** 1994. Structure and function of the b/HLH/Z domain of USF. EMBO J. **13:**180–189.
10. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
11. **Fink, G. R.** 1970. The biochemical genetics of yeasts. Methods Enzymol. **17:**59–78.
12. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425–1426.
13. **Guarente, L., and M. Ptashne.** 1981. Fusion of *Escherichia coli lacZ* to the cytochrome c gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78:**2199–2203.
14. **Gyuris, J., E. Golemis, H. Chertkov, and R. Brent.** 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **74:**205–214.
15. **Hanes, S. D., and R. Brent.** 1989. DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue. Cell **57:**1275–1293.
16. **Hieter, P., D. Pridmore, J. H. Hegemann, H. Thomas, R. W. Davis, and P. Philippsen.** 1985. Functional selection and analysis of yeast centromeric DNA. Cell **42:**913–921.
17. **Higgins, D. G.** 1994. CLUSTAL V: multiple alignment of DNA and protein sequences. Methods Mol. Biol. **25:**307.
18. **Hodgson, C. P., and R. Z. Fisk.** 1987. Hybridization probe size control: optimized "oligolabelling". Nucleic Acids Res. **15:**6295.
19. **Hoffman, C. S., and F. Winston.** 1987. A ten minutes DNA preparation from yeast releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
20. **Isnard, A. D., D. Thomas, and Y. Surdin-Kerjan.** 1996. The study of methionine uptake in *Saccharomyces cerevisiae* reveals a new family of amino acid permeases. J. Mol. Biol. **262:**473–484.
21. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
22. **Korch, C., H. A. Mountain, and A. S. Bystrom.** 1991. Cloning, nucleotide sequence and regulation of *MET14*, the gene encoding the APS kinase of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **228:**96–108.
23. **Kuras, L., R. Barbey, and D. Thomas.** 1997. Assembly of a bZIP/bHLH transcription activation complex: formation of the yeast Cbf1/Met4/Met28 complex is regulated through Met28 stimulation of Cbf1 DNA binding. EMBO J. **16:**2447–2451.
24. **Kuras, L., H. Chérest, Y. Surdin-Kerjan, and D. Thomas.** 1996. A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. EMBO J. **15:**2519–2529.
25. **Kuras, L., and D. Thomas.** 1995. Functional analysis of Met4, a yeast transcriptional activator responsive to *S*-adenosylmethionine. Mol. Cell. Biol. **15:**208–216.
26. **Kuras, L., and D. Thomas.** 1995. Identification of the yeast methionine biosynthetic genes that require the centromere factor 1 for their transcriptional activation. FEBS Lett. **367:**15–18.
27. **Rothstein, R. J.** 1983. One step gene disruption in yeast. Methods Enzymol. **101:**202–211.
28. **Sherman, F., G. R. Fink, and J. B. Hicks (ed.).** 1979. Methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
30. **Thomas, D., H. Cherest, and Y. Surdin-Kerjan.** 1989. Elements involved in *S*-adenosylmethionine mediated regulation of the *Saccharomyces cerevisiae MET25* gene. Mol. Cell. Biol. **9:**3292–3298.
31. **Thomas, D., I. Jacquemin, and Y. Surdin-Kerjan.** 1992. MET4, a leucine zipper protein, and centromere-binding factor I, are both required for transcriptional activation of sulfur metabolism in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**1719–1727.
32. **Thomas, D., L. Kuras, R. Barbey, H. Cherest, P. L. Blaiseau, and Y. Surdin-Kerjan.** 1995. Met30, a yeast transcriptional inhibitor that responds to *S*-adenosylmethionine, is an essential protein with WD40 repeats. Mol. Cell. Biol. **15:**6526–6534.
33. **Thomas, P. S.** 1980. Hybridization of denatured DNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.
34. **Vallee, B. L., J. E. Coleman, and D. S. Auld.** 1991. Zinc fingers, zinc clusters and zinc twist in DNA binding protein domains. Proc. Natl. Acad. Sci. USA **88:**999–1003.