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MET4, a Leucine Zipper Protein, and Centromere-Binding Factor 1 Are Both Required for Transcriptional Activation of Sulfur Metabolism in *Saccharomyces cerevisiae*

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Inactivation of the centromere-binding factor 1 (*CBF1*) gene results in yeast strains that require methionine for growth. This auxotrophy is due to the inability of such strains to concentrate and assimilate sulfate from the medium. Northern (RNA) blot experiments reveal that the CBF1 protein is required for full induction of *MET25* and *MET16* gene transcription. However, we show that induction of the sulfate assimilation pathway is not achieved solely by CBF1. This induction also requires the integrity of a positive *trans*-acting factor, encoded by the *MET4* gene. The *MET4* gene was cloned, and its sequence reveals that it encodes a protein related to the family of the bZIP transcriptional activators. Evidence that MET4 is a transcriptional activator was provided by demonstrating that DNA-bound LexA-MET4 fusion proteins stimulate expression of a nearby promoter. The use of LexA-MET4 fusion proteins also reveals that the leucine zipper of MET4 is required for the recognition of the *MET25* promoter. Moreover, an 18-bp fragment of the *MET25* 5' upstream region was found to confer *S*-adenosylmethionine-dependent regulation of a fusion gene. This regulation was shown to depend on both MET4 and CBF1. The obtained results suggest that the binding of CBF1 to its cognate sequences increases the ability of MET4 to stimulate transcription of the *MET* genes.

Reductive assimilation of sulfate and synthesis of organic sulfur compounds (cysteine, methionine, and *S*-adenosylmethionine [AdoMet]) constitute a single metabolic pathway in *Saccharomyces cerevisiae*. All of the structural genes governing this pathway are subjected not to the general amino acid control but to a specific negative regulation. This regulation, resulting from the increase in intracellular concentration of AdoMet, acts mainly by decreasing the level of transcription of these coregulated genes (40). A deletion analysis of the 5' upstream region of the *MET25* gene (coding for homocysteine synthase) has led to the identification of a *cis*-acting sequence (5'-TCACGTGA-3') that is critical for transcriptional control (40). The same sequence is also found in the 5' flanking region of the coregulated genes *MET2*, *MET3*, *MET8*, *MET14*, *MET16*, and *SAM2* (21, 39), suggesting its implication in proper regulation of *MET* genes.

Unexpectedly, this sequence had been identified previously as a component of another unrelated biological process, DNA segregation. Indeed, this sequence, called CDEI (for centromere DNA element I), is completely conserved in all known yeast centromeres, and mutations in this element impair centromere function (17). A unique protein, called CBF1 or CPF1, has been shown to bind specifically to the CDEI element, and sequencing of the *CBF1* gene has revealed that this protein is related to the helix-loop-helix protein family (1a, 5, 28). As expected, a *cbf1* null mutant exhibits defects in chromosomal segregation; it also displays a nutronal requirement for methionine (1a, 5). Following our analysis of the *MET25* upstream region and considering the fact that CBF1 binds in vitro to the *MET25* promoter (1, 29), the methionine auxotrophy of a *cbf1* mutant seemed to be explained by the implication of the CBF1 factor in sulfur gene expression. However, in a recent study, Mellor et al. (29), on the basis of Northern (RNA) blot experiments, were not able to confirm this model and therefore failed to explain the phenotype exhibited by a *cbf1* mutant.

We have thus initiated work intended to determine which enzymatic defects are responsible for the nutritional requirement exhibited by a *cbf1* mutant. Results presented here clearly explain the methionine auxotrophy of a *cbf1* mutant and confirm the involvement of CBF1 in transcriptional activation of sulfate metabolism. However, these results do not account for a model in which induction of this pathway is achieved solely by CBF1. A systematic analysis of Met$^-$ mutants allowed us to determine that gene *MET4* encodes a second factor necessary for proper induction of the sulfate assimilation pathway. Cloning of this gene revealed that it encodes a protein related to the leucine zipper protein family. Results of experiments using MET4 and CBF1-LexA hybrid proteins and *MET25-CYC1-lacZ* fusion plasmids suggest that the binding of CBF1 to its sites could assist the MET4 protein in stimulating transcription.

MATERIALS AND METHODS

Strains, media, and microbiological techniques. *Escherichia coli* HB101 and JM103 were used as hosts for plasmid maintenance. *S. cerevisiae* strains used in this work are listed in Table 1. To grow *E. coli*, we used media described by Maniatis et al. (26). For *S. cerevisiae*, YPG and YNB media were as described by Sherman et al. (36). *E. coli* was transformed according to Cohen et al. (9). *S. cerevisiae* was transformed after lithium chloride treatment as described by Ito et al. (20). Genetic crosses, sporulation, dissection, and scoring of nutritional markers were done as described by Sherman et al. (36).

Plasmid constructions. For the subcloning experiments, we used the shuttle vectors pEMBLYe23 (2) and pRS316 (37). The *S. cerevisiae* genomic library that allowed cloning of the

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TABLE 1. Yeast strains

Strain[a]	Genotype	Source
CC359-OL2	*his3 ura3 leu2*	H. Cherest
CC471-1D	*leu2 ura3 met4*	H. Cherest
CC486-6B	*ade2 met4 sam1::URA3*	This study
CC489-7A	*his3 leu2 met4 sam2::URA3*	This study
CC561-1A	*leu2 ura3 trp1*	This study
CC561-7D	*his3 leu2 ura3 trp1 cbf1::URA3*	This study
CD106	*MAT***a** *his3 leu2 ura3 ade2 trp1 met4::TRP1*	This study
R31-3B	*MAT***a** *his3 leu2 ura3 lys2 trp1 ade2 cbf1::TRP1*	R. Baker
W303-1A	*MAT***a** *his3 leu2 ura3 ade2 trp1*	R. Rothstein
X2180-1A	*MAT***a**	Yeast Genetic Stock Center

[a] Strains CC561-1A and CC561-7D are two spores issuing from the same cross; strain CD106 was obtained by a one-step disruption of the *MET4* gene in strain W303-1A.

MET4 gene was constructed by inserting the product of a partial *Sau*3A digest of chromosomal DNA from strain X2180-1A in the *Bam*HI site of plasmid pEMBLYe23. The effector plasmids expressing LexA-MET4 fusion proteins were constructed as follows. Plasmid pLexM4-1, expressing LexA (1 to 87)-MET4 (15 to 666), was created by inserting the *Eco*RI-*Bam*HI fragment of pM4-4 into the *Eco*RI-*Bam*HI sites of pSH2-1 (16); plasmid pLexM4-2, expressing LexA (1 to 87)-MET4 (15 to 558), was created by inserting the *Eco*RI-*Sal*I fragment of pM4-4 into the *Eco*RI-*Sal*I sites of pSH2-1; pLexM4-3, expressing LexA (1 to 87)-MET4 (15 to 616), was created by inserting the *Eco*RI-*Cla*I fragment of pM4-4 into the *Eco*RI-*Bam*HI sites of pSH2-1 after the *Cla*I and *Bam*HI sites were filled in with Klenow enzyme. The plasmid expressing a LexA (1 to 87)-CBF1 (10 to 351) fusion protein was constructed as follows. Plasmid pSH2-1 was first linearized by *Eco*RI, treated with mung bean nuclease to remove the 5′ overhanging ends, and then cut by *Bam*HI. The *Hin*dIII (filled in with the Klenow enzyme)-*Bgl*II fragment of plasmid pSP65-1, which bears the *CBF1* gene (28), was ligated to plasmid pSH2-1 treated as described above. This in-frame construction was checked by sequence analysis. Fusions of *MET25* regulatory regions to a *CYC1-lacZ* chimera were obtained by inserting *MET25* promoter elements into the *Xho*I site of plasmid pLG670Z (15). All constructions were confirmed by sequence analysis.

DNA sequencing. The *Xba*I-*Xba*I fragment of pM4-4 was subcloned in two orientations into bacteriophage M13mp18, and systematic deletion subclones were generated as described by Thomas and Surdin-Kerjan (41). Corresponding single-stranded phage DNAs were sequenced with a Pharmacia T7 sequencing kit. Sequence analyses were performed by using the Bisance service (10).

Northern experiments. Northern blotting was performed as described by Thomas (42) with total cellular RNA extracted from yeast cells as described by Hoffman and Winston (18).

Enzymatic assays. Yeast cells were grown to 10^7 cells per ml in 10 ml of the appropriate minimal synthetic medium. Cell extracts were made as described by Thomas et al. (40) in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. After centrifugation for 15 min at 10,000 × *g*, the crude extract was used for enzymatic assays.

For homocysteine synthase assay, the reaction mixture contained, in a final volume of 0.1 ml, 100 mM Tris-HCl (pH 8.0), 0.2 mM pyridoxal phosphate, 20 mM *O*-acetyl-L-homoserine, and the cell extract containing 3 to 50 μg of protein. Four different protein concentrations were assayed for each cell extract. Incubation was for 15 min at 30°C, and the homocysteine formed was measured as described by Kredich and Tomkins (22), using a standard curve made with known concentrations of L-cysteine. For the ATP sulfurylase assay, we used a modification of the method described by de Vito and Dreyfuss (11). The reaction mixture contained, in a final volume of 0.5 ml, 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 5 mM sodium molybdate, and cell extract containing 0.5 to 1 mg of protein. Three protein concentrations were assayed for each cell extract. Addition of pyrophosphatase was not necessary because of the high content of the endogenous enzyme in cell extracts. Incubation was for 30 min at 37°C and was stopped by addition of 0.5 ml of 10% trichloroacetic acid. After centrifugation (5 min at 10,000 × *g*), the phosphate formed from ATP and present in 0.1 ml of the supernatant was measured. 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) reductase was assayed as described by Thomas et al. (39). Sulfate permease was assayed as described by Breton and Surdin-Kerjan (3). β-Galactosidase was assayed as described by Miller (30). Protein concentrations were estimated by the method of Lowry et al. (25).

Nucleotide sequence accession number. The nucleotide sequence of the *MET4* gene shown in Fig. 2 has been deposited with GenBank under accession number M84455.

RESULTS

CBF1 is required for normal transcription of *MET16* and *MET25*. The phenotype of a *cbf1*-deficient strain suggests that CBF1 is involved in both chromosomal segregation and activation of transcription. However, direct evidence for the second function was not provided. As a *cbf1* null mutant grows only in the presence of organic sulfur sources, we searched first for the physiological bases of this auxotrophy. We thus measured in a *cbf1* null mutant the level of enzymes that allow the assimilation of sulfate. This was done in two isogenic strains, CC561-7D (*cbf1::URA3*) and CC561-1A (*CBF1*), which were both grown on 0.2 mM DL-homocysteine as the sulfur source. As shown in Table 2, inactivation of the *CBF1* gene does not affect the level of ATP sulfurylase or sulfite reductase but results in a threefold decrease of homocysteine synthase activity (encoded by the *MET25* gene) and greatly decreases both PAPS reductase (encoded by the *MET16* gene) and sulfate permease activities. Such enzymatic deficiencies explain why a *cbf1*-deficient strain requires the presence of organic sulfur sources to grow. Northern analysis of RNA extracted from a *cbf1* null mutant confirms that the decrease of homocysteine synthase activity correlates with a reduction in the *MET25* transcript level compared with the wild-type level and that the lack of PAPS reductase activity corresponds to an undetectable transcription of the *MET16* gene (Fig. 1). These results clearly show that the CBF1 protein is required for full induction of at least the *MET25* and *MET16* genes.

The *MET4* gene is required for induction of the sulfate assimilation pathway. Investigations of different *met* mutations showed the predominant role of the *MET4*-encoded product in the sulfate assimilation pathway. As shown in Table 3, a *met4* mutant strain is devoid of all enzymatic functions necessary to assimilate sulfate. Genetic lesions inducing multiple losses of enzymatic activities from this pathway have already been reported (27) and recently analyzed (39), but the *met4* mutation is the only known mutation

TABLE 2. Specific activities of enzymes involved in the sulfate assimilation pathway assayed in a wild-type and a *cbf1*-disrupted strain

Strain[a]	Relevant genotype	Activity[b]				
		Homocysteine synthase	ATP sulfurylase	PAPS reductase	Sulfite reductase	Sulfate permease
CC561-1A	*CBF1*	431	129	11.5	1.5	16
CC561-7D	*cbf1::URA3*	156	145	0	1.2	<1

[a] Grown in minimal medium plus 0.2 mM DL-homocysteine.

[b] Expressed in nanomoles per minute per milligram of protein except PAPS reductase activity, which is expressed in nanomoles per hour per milligram of protein. Data are means of two experiments.

which results in the lack of all of these activities. Northern analysis of RNA extracted from a *met4* mutant strain grown under derepressing conditions (0.05 mM L-methionine) reveals that these deficiencies are due to a lack of transcription of the corresponding genes (Fig. 1). The *MET4* gene thus encodes a *trans*-acting factor responsible for the transcriptional activation of inorganic sulfur metabolism. It must be noted that the MET4 protein appears to govern only the expression of genes belonging to the sulfate assimilation pathway, not those implicated in further metabolism of the sulfur amino acids. Indeed, whereas a *met4* mutant fails to grow on all inorganic sulfur sources, this strain is able to grow on organic sulfur sources such as homocysteine, cysteine, methionine, and AdoMet. In addition, the fact that strains CC486-6B and CC489-7A (*met4 sam1::URA3* and *met4 sam2::URA3*, respectively) do not display an AdoMet requirement rules out the possibility that MET4 is required for proper expression of one of the AdoMet synthetase genes.

Cloning and sequencing of the *MET4* gene. The *MET4* gene was cloned by complementing the methionine requirement of strain CC471-1D. This strain was transformed with the pEMBLYe23-based library described in Materials and Methods. Among 10,000 Ura^+ transformants tested, 2 strains able to grow in the absence of methionine were found. These clones harbored identical plasmids with a 8,200-bp insert (Fig. 2). The *MET4* gene maps on the left arm of the chromosome XIV, near the *LEU4* and *POL1* (*CDC17*) genes (31). These two genes have been cloned (6, 33), and comparison of the restriction maps reveals that the left part of the pM4-1 insert overlaps by about 3.5 kbp plasmid pTSC2, which bears the *LEU4* gene (6), while the right part of pM4-4 overlaps by about 3.2 kbp the *POL1*-bearing plasmid (33). Moreover, Chang et al. (7) showed that replacement of a genomic region containing the *LEU4* gene and the equivalent of the right part of plasmid pM4-1 (up to the *Eco*RI site) by the *HIS3* gene led to a methionine auxotrophic strain. This mutation was shown to be allelic to the previously reported *met4* mutations. All of these results demonstrate that the cloned DNA carried by plasmid pM4-1 is *MET4*. The organization of the *MET4* locus is depicted in Fig. 2.

The 2,500-bp *Xba*I-*Xba*I fragment of plasmid pM4-4 was sequenced as described in Materials and Methods. In addition, the *Eco*RI-*Sph*I fragment of plasmid pM4-1 was sequenced to verify the region overlapping the *LEU4* gene. Comparison of the *MET4* and *POL1* sequences revealed that the 5′ noncoding region of *POL1* reported by Pizzagalli et al. (33) contains a duplication of 4 bp (nucleotides TATG, positions −640 to −648 as numbered in that report) which was not found in the sequence of pM4-4.

Analysis of the sequenced region reveals the presence of a unique open reading frame of 1,998 bp which is separated from the *LEU4* open reading frame by 1,010 bp and which ends 353 nucleotides upstream of the *POL1* open reading frame (Fig. 3).

The predicted MET4 protein bears a leucine zipper preceded by an unusual basic domain. The predicted MET4 polypeptide is composed of 666 amino acids with a calculated molecular mass of 73,400 Da. This protein is substantially hydrophilic, with equal amounts of positively (14%) and negatively (14%) charged amino acids. Asparagine and serine residues predominate (13% of each) and lie mostly in the NH_2 part of the polypeptide. Such a bias in the amino acid composition was also observed in other yeast transcriptional activators such as PHO2 (35) and GCR1 (19). In addition, the MET4 protein is free of cysteine residues; it was pointed out in a previous report (39) that a low content of cysteine residues (<0.3 cysteine per 100 amino acids) is a general feature shared by proteins implicated in sulfur amino acid metabolism in *S. cerevisiae* as well as in *E. coli*. As shown in Fig. 4, the predicted MET4 protein bears at its carboxy terminus a repeat of five leucine residues spaced every seven amino acids. Furthermore, this leucine repeat is immediately preceded by a basic region. A similar molecular organization was found in some regulatory proteins from higher eucaryotes as well as from fungi by Landshulz et al. (24), who proposed that such proteins (called bZIP, for basic region-leucine zipper) interact with DNA through this bipar-

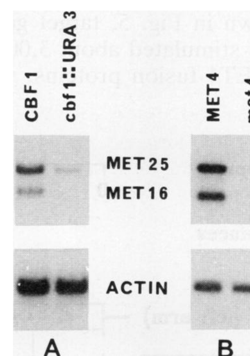

FIG. 1. Northern blot analysis of steady-state *MET16* and *MET25* RNA levels for a *cbf1::URA3* strain (A) and for a *met4* strain (B). The strains used were CC561-1A (*CBF1*), CC561-7D (*cbf1::URA3*), CC359-OL2 (*MET4*), and CC471-1D (*met4*). Total RNA was extracted from exponentially growing cells. Strains CC561-1A and CC561-7D were grown in the presence of 0.2 mM homocysteine, and strains CC359-OL2 and CC471-1D were grown in the presence of 0.05 mM L-methionine. Total RNA (10 μg per lane) was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. The transferred RNAs were hybridized simultaneously to the radioactive *Nsi*I-*Nsi*I fragments from the *MET16* gene and to the *Xba*I-*Eco*RV fragment from the *MET25* gene (39). The actin probe was used as a control for the amounts of RNA loaded.

TABLE 3. Specific activities of enzymes involved in the sulfate assimilation pathway, assayed in a wild-type and a *met4* mutant strain

Strain[a]	Relevant genotype	Activity[b]				
		Homocysteine synthase	ATP sulfurylase	PAPS reductase	Sulfite reductase	Sulfate permease
CC359-OL2		464	139	23.4	1.4	15
CC471-1D	*met4*	37	1.6	0	0.2	2.1

[a] Grown in minimal medium plus 0.05 mM L-methionine.
[b] Expressed in nanomoles per minute per milligram of protein except PAPS reductase activity, which is expressed in nanomoles per hour per milligram of protein. Data are means of two experiments.

tite motif. Physical and genetic experiments confirm that the leucine zipper allows the formation of stable dimer while the basic region is responsible for specific DNA binding (32, 38). The algorithm of Garnier et al. (14) predicts that the region of the MET4 protein (from residues 591 to 647) containing both the basic motif and the leucine repeat could fold into an α-helix secondary structure. In particular, this region is free of helix-destabilizing residues (proline and glycine). A comparison of the leucine zipper motif of MET4 with other known leucine zipper motifs, using the alignment proposed by Vinson et al. (43), is illustrated in Fig. 4. It is interesting to note that the highest homology is found between the *MET4*-encoded product and the transcription factor CYS3 from *Neurospora crassa*, which encodes a positive-acting regulatory protein of the sulfur metabolism in *N. crassa* (13). However, the similarity between the two proteins is restricted to the regions surrounding the leucine zipper motif (Fig. 4).

MET4 but not CBF1 can activate transcription. To test whether the MET4 and CBF1 proteins could activate transcription, we constructed MET4 and CBF1 fusion proteins that contain the DNA-binding domain of the bacterial repressor *lexA*. The fusion proteins were expressed under the control of an *ADH1* promoter, using plasmid pSH2-1 (16). The ability of LexA-MET4 and LexA-CBF1 fusion proteins to activate transcription was tested by using a target plasmid, pSH18-34 (16a), bearing a *GAL1-lacZ* reporter gene that carried *lexA*-binding sites upstream of the transcription start site. As shown in Fig. 5, target genes carrying *lexA*-binding sites were stimulated about 3,000-fold by the three different LexA-MET4 fusion proteins, while those lacking *lexA*-binding sites were not stimulated. As it is the case for the previously described bZIP yeast activators, the leucine zipper and nearby basic domain of MET4 appear not to be required for the activating functions. By contrast, strong activation of the *MET25* gene by the LexA-MET4 fusion proteins requires that hybrids contain the carboxy terminus of MET4 (Fig. 5). The finding that pLexM4-1 alone complements the methionine auxotrophy of a *met4*-disrupted strain confirms this result. However, weak (about threefold) stimulation of homocysteine synthase is measured in strains transformed with pLexM4-3 that express a hybrid bearing the basic domain but not the leucine zipper of MET4. In addition, induction of *MET25* is decreased by the presence of *lexA* operators in cells, reflecting a trapping effect of the hybrid proteins by these DNA sequences. Unlike the LexA-MET4 fusion proteins, the LexA-CBF1 fusion protein cannot stimulate the *GAL1-lacZ* gene containing upstream *lexA* operators although the fusion protein expressed by plasmid pLexCBF1 is functional, as demonstrated by the methionine prototrophy of a *cbf1*-disrupted strain transformed by this plasmid (Fig. 5). These results strongly suggest that the DNA-binding protein CBF1 lacks activating functions.

AdoMet-dependent activation of a *MET25-CYC1-lacZ* gene fusion. Construction of deleted alleles of the 5′ upstream region of the *MET25* gene has led to the identification of the *cis*-acting sequences required for transcriptional activation (40). These sequences have been localized around nucleotide −300, a region that contains two copies of the CDEI element. To determine whether sequences of the −300 region can activate the transcription of a heterologous gene, we inserted oligonucleotides corresponding to these se-

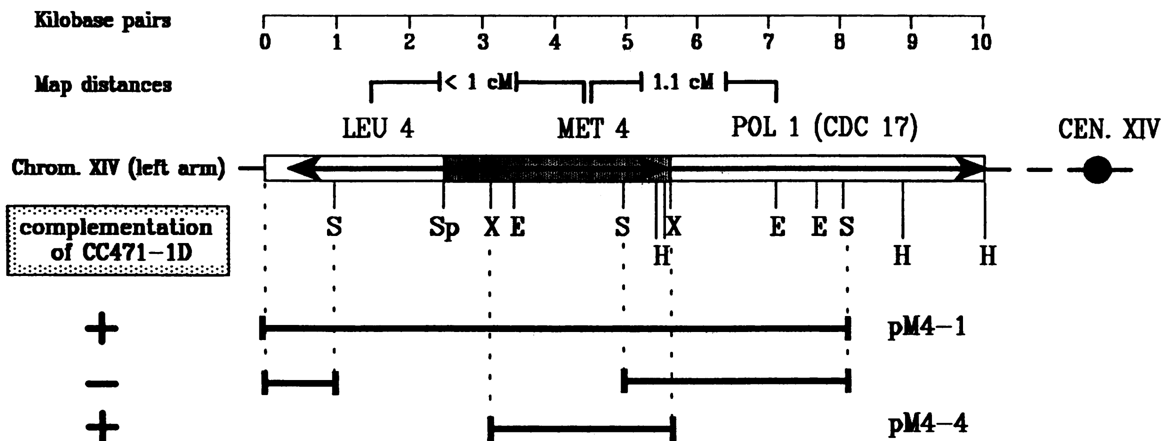

FIG. 2. Physical map of the *LEU4-MET4-POL1* region. The fragments subcloned in plasmid pEMBLYe23 or pRS316 as well as their abilities to complement the mutation of strain CC471-1D are shown. Genetic distances (in centimorgans [CM]) are from Mortimer et al. (31) and Chang et al. (6). Restriction sites: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sp, *Sph*I; X, *Xba*I.

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                                                                                    CAG CAA CAG -1002
                                                                     <--- LEU4       L   L   L
AAT AAT ACT CTC TTT AAC CAT gattgttccttgtctttttgtaaggaaaaagtctacagtaaacttctagtgtgagaatcctttttttttcttcaaaaaataccct -897
 I   I   S   E   K   V   M
ttgaaagaactgagtcacttcacagtaagtttctttttactggtcaagttgaaaaataaccaataaatgattatggaaagaacgttgaaagaggagagaggctgacccaaga -785
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ttagtcaactcctgaaggtcttcttgcctgtccgtcaactgtttagacagactctcgtcaataaagcgcacttctgataagcacttttattcctttttttccactgtgaacg   -1
MET4 --->
ATG AAG CAG GAG CAG TCC CAC GAA GGC GAC TCA TAC AGC ACG GAA TTC ATA AAT CTC TTT GGC AAA GAT ACC GCA ACA CAC CCT   84
 M   K   Q   E   Q   S   H   E   G   D   S   Y   S   T   E   F   I   N   L   F   G   K   D   T   A   T   H   P

AGC AGC AAC AAC GGT GCT AAT AAT AAT GGC ATG GGG AGC ACG AAC TCG TTG GAC CAG TTT GTG GCA ACA GCC TCA TCG TCA TCT  168
 S   S   N   N   G   A   N   N   N   G   M   G   S   T   N   S   L   D   Q   F   V   A   T   A   S   S   S   S

TCT CTG GTG ACC AGC AGC GAG AAT AGG CGC CCC CTA ATA GGT GAC GTT ACC AAT AGG GGC AAC ACT AAC CTA TAT GAC CAC GCT  252
 S   L   V   T   S   S   E   N   R   R   P   L   I   G   D   V   T   N   R   G   N   T   N   L   Y   D   H   A

GTC ACG CCA GAA ATA CTC TTA GAA CAG TTG GCC TAC GTG GAT AAC TTC ATA CCA TCT CTG GAT AAC GAG TTC TCT AAT GTG GAT  336
 V   T   P   E   I   L   L   E   Q   L   A   Y   V   D   N   F   I   P   S   L   D   N   E   F   S   N   V   D

TGG AAT GTG AAT ACC ACC CAT AAT AAT GCA AAC AAT AAT GGC GCG GAC ACT TTC AGC AGC ATA AAT GCA AAT CCT TTT GAC TTG  420
 W   N   V   N   T   T   H   N   N   A   N   N   N   G   A   D   T   F   S   S   I   N   A   N   P   F   D   L

GAT GAA CAA CTA GCC ATT GAG TTG AGT GCG TTT GCC GAC GAT TCT TTC ATC TTC CCA GAC GAG GAT AAG CCG AGC AAT AAC AAT  504
 D   E   Q   L   A   I   E   L   S   A   F   A   D   D   S   F   I   F   P   D   E   D   K   P   S   N   N   N

AAC AAC AGC AAT AAT GGT AAT GAC GAC CAT AGC AAC CAC GAC GTA TTG CAT GAG GAC CCT TCT ACC AAT AAT AGA CAA AGA AAT  588
 N   N   S   N   N   G   N   D   D   H   S   N   H   D   V   L   H   E   D   P   S   T   N   N   R   Q   R   N

CCT CAC TTC TTG ACT CAA AGA AGG AAT ACT TTC CTA ACT TCC CAA TAC GAC CAA TCA AAG TCT CGA TTT TCG TCC AAA AAC AAA  672
 P   H   F   L   T   Q   R   R   N   T   F   L   T   S   Q   Y   D   Q   S   K   S   R   F   S   S   K   N   K

AGA AAT GGC AAT AAC GGC GAA ACA AAC AAC TTT GGC GAC AAT ATG CAA AAT AAC CAT CCT TTT GAG CCA AAC TTT ATG GGA AGC  756
 R   N   G   N   N   G   E   T   N   N   F   G   D   N   M   Q   N   N   H   P   F   E   P   N   F   M   G   S

CCT TCT CAG TTT CCC GCT GAC GCA ACT AAT ATG ACA TCA ATC GAC CAT GGC GGC TTC ACA AAT GTT GAC ATT ACA TCA ACT GAG  840
 P   S   Q   F   P   A   D   A   T   N   M   T   S   I   D   H   G   G   F   T   N   V   D   I   T   S   T   E

AAC AAT ACT ACC GGT GAC AAT GGA GTG GAT GCG CTA TCA AAT CTA CTA CAT AGG ACA ACA CAC ACA CCG AAC CGC TCC TCC CCC  924
 N   N   T   T   G   D   N   G   V   D   A   L   S   N   L   L   H   R   T   T   H   T   P   N   R   S   S   P

CTA AGC AAT GTC ACT TCT GCT CAA AAT TCC TCT TCA CAA CAA CGA AAA CAT TCG GAA AGC AAA GTC GAT AGT AAC AGC GAT AAT 1008
 L   S   N   V   T   S   A   Q   N   S   S   S   Q   Q   R   K   H   S   E   S   K   V   D   S   N   S   D   N

AAC AGC TCC AAC AAA GCC CCC AAT ATA ACT GTT CCT GAC TAT TCA ATT ATA CCA ACC TCT GTT TTA GTA ACT CTA TTA CCG AGG 1092
 N   S   S   N   K   A   P   N   I   T   V   P   D   Y   S   I   I   P   T   S   V   L   V   T   L   L   P   R

GTC AAC GTG CCC AAC GGC GCA TAT AAC TCG TTG ATC AGC GCG GGA TTT GAC AAT GAT CAA ATA GAT GCT ATA GCC GCA ATA ATG 1176
 V   N   V   P   N   G   A   Y   N   S   L   I   S   A   G   F   D   N   D   Q   I   D   A   I   A   A   I   M

GCG TAT CAT CAT CAA AAA AAG ATT AGG GAA AAT AAC AGT AAT AAT AAT AAA AAC ATC AAC ACC AAT GAC AGT CAA GAG GCA CCC 1260
 A   Y   H   H   Q   K   K   I   R   E   N   N   S   N   N   N   K   N   I   N   T   N   D   S   Q   E   A   P

ATT CTA AAA AAC ATC AAC GAA CTT TTA AGT GTC TTA ATA CCA CCC TCT CCG GCT GAA ACT GCT GCG CCA ACT ACC TTA TCA ACG 1344
 I   L   K   N   I   N   E   L   L   S   V   L   I   P   P   S   P   A   E   T   A   A   P   T   T   L   S   T

TCG CCT TCG TTC AAT GAG CAC GGT GTA GTA GCA GAG GCT TCT TTT CTA AGC TCC ATT TTG GAA CTG GGC ATA AAG CAT CCA AAA 1428
 S   P   S   F   N   E   H   G   V   V   A   E   A   S   F   L   S   S   I   L   E   L   G   I   K   H   P   K

AGT AAT AAT ATT CAC AAT CAA CGA CAA CCT TCA CGA AAC GAT CAT AAA ATA TCA AGA GAG AGT GAC GGT AAC AAT GGA AAC GAT 1512
 S   N   N   I   H   N   Q   R   Q   P   S   R   N   D   H   K   I   S   R   E   S   D   G   N   N   G   N   D

AAT GTC CAT CAT AAT AAC GCT GTT ATT AAG TCA AGT ACG ACG CGT GGA GAC GAA ATT GCC AAG ATA CGA TCC GAG CCA ACT TTA 1596
 N   V   H   H   N   N   A   V   I   K   S   S   T   T   R   G   D   E   I   A   K   I   R   S   E   P   T   L

AAT GCA AGT TCT TCT GAT CAC AAG GAA AAT AGT TTA AAA AGA TCA CAC TCC GGA GAT TTG AAA AAT AAA AAA GTA CCC GTC GAC 1680
 N   A   S   S   S   D   H   K   E   N   S   L   K   R   S   H   S   G   D   L   K   N   K   K   V   P   V   D

CGC AAG TAT TCT GAT AAT GAA GAC GAT GAA TAT GAC GAT GCA GAT TTA CAC GGC TTT GAA AAG AAG CAA CTG ATC AAG AAA GAG 1764
 R   K   Y   S   D   N   E   D   D   E   Y   D   D   A   D   L   H   G   F   E   K   K   Q   L   I   K   K   E

TTA GGG GAC GAC GAT GAA GAT TTA TTG ATA CAG TCG AAA AAA TCT CAT CAA AAA AAA AAA CTA AAG GAA AAG GAG TTA GAA TCA 1848
 L   G   D   D   D   E   D   L   L   I   Q   S   K   K   S   H   Q   K   K   K   L   K   E   K   E   L   E   S

TCG ATA CAT GAA CTG ACC GAA ATT GCA GCA TCC TTA CAA AAA CGG ATA CAT ACG TTA GAA ACG GAA AAC AAG CTT TTA AAG AAT 1932
 S   I   H   E   L   T   E   I   A   A   S   L   Q   K   R   I   H   T   L   E   T   E   N   K   L   L   K   N

TTA GTT CTG AGT AGC GGT GAA ACG GAA GGA ATA AAA AAA GCT GGA AAG CTT AAA GAA GCA AAT TTT TGA  gaaggttcagaaagaataa 2020
 L   V   L   S   S   G   E   T   E   G   I   K   K   A   G   K   L   K   E   A   N   F   *

aataacagactatacagtttaattatatatatatatatatatacgtccatttttcttaaagaaatataactttttttcttgtactgcctgcaatctctattcttcattcatca 2132
cacatctattcaaacgcgttaaaatttttttgttagcctataagtaaaacgcgtcgcgtgtatccaattttactgaattaaattctttactcattggatgatatatatcttc 2244
ttggtctagacaacgaacacataccaattcagactcacgagaaaaaaaactgtcttcactctgctattagaaaccaaatagtatcaaggcataaaataaacagtggaaa   2353
ATG TCT TCT AAA AGT GAA                                                                                          2371
 M   S   S   K   S   E   POL1 --->
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *MET4* region. The noncoding strands of genes *MET4* and *POL1* are shown. The nucleotide sequence is numbered from nucleotide 1 of the presumed initiation codon of *MET4*. The CDEI element found in the 5′ noncoding region of *MET4* is underlined, as is the region of the MET4 protein that contains the leucine zipper motif.

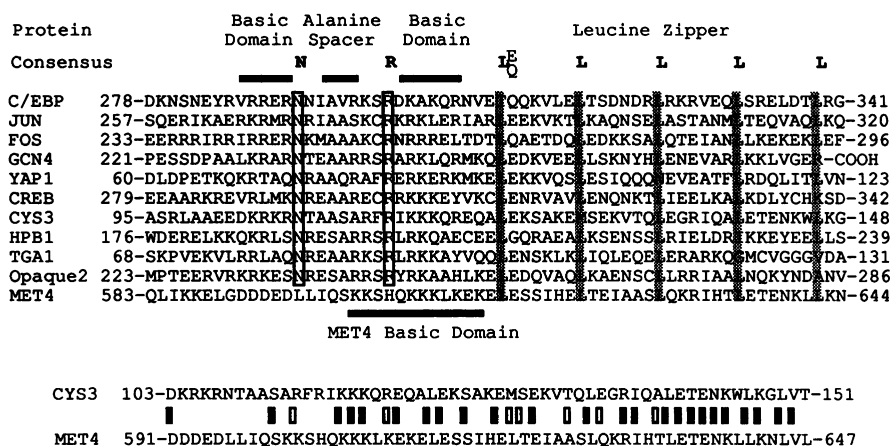

FIG. 4. Comparison of the MET4 basic leucine zipper motif with other known bZIP motifs. The MET4 basic leucine zipper region is compared with the other known bZIP motifs aligned according to Vinson et al. (43). The leucine residues are shaded; invariant asparagine and arginine residues are boxed; basic clusters and the alanine spacer are indicated by solid bars over the sequences. The MET4 stretch of basic residues is indicated by a solid bar under the sequence. Proteins are identified at the left. Polypeptide sequences, designated according to the single-letter code, are numbered in relation to the NH_2 terminus. In addition, alignment of the regions surrounding the leucine zipper motifs of CYS3 and MET4 is shown. Black boxes indicate identities; white boxes indicate conservative replacements.

quences in the 5′ upstream region of a *CYC1-lacZ* gene fusion (15). In each case, oligonucleotides replaced the upstream activation element of the *CYC1* gene. The *MET25-CYC1-lacZ* fusion constructs were used to transform a wild-type strain, a *met4*-disrupted strain, and a *cbf1*-disrupted strain. As shown in Fig. 6, plasmid pLGC3, which contains a CDEI element alone, failed to express more β-galactosidase activity than did the parent vector pLG670Z, regardless of genetic background. The inability of the pLGC3 to express any detectable β-galactosidase activity agrees with the results of Buchman and Kornberg (4), who showed that a fragment of the *GAL2* promoter containing a CDEI element is not able to efficiently stimulate transcription from their tester promoter. An 18-bp oligonucleotide (5′-TGGCAAATGGCACGTGAA-3′), comprising a CDEI element and the preceding 9 bp (spanning nucleotides −310 to −294 of the *MET25* promoter), was inserted into pLG670Z to make pLGF12. Under derepressing conditions (0.05 mM L-methionine), this construct expressed about 150 U of β-galactosidase in a wild-type genetic background. The same level of activation was obtained with plasmid pLGB3, which contains a duplication of the 18-bp oligonucleotide. Growth under repressing conditions (1.0 mM L-methionine) leads to a 10-fold decrease of the β-galactosidase activities expressed from plasmids pLGF12 and pLGB3, resulting in the level of activity expressed by the parent vector, pLG670Z. Furthermore, all of the *MET25-CYC1-lacZ* gene fusion fails to express any significant β-galactosidase activity

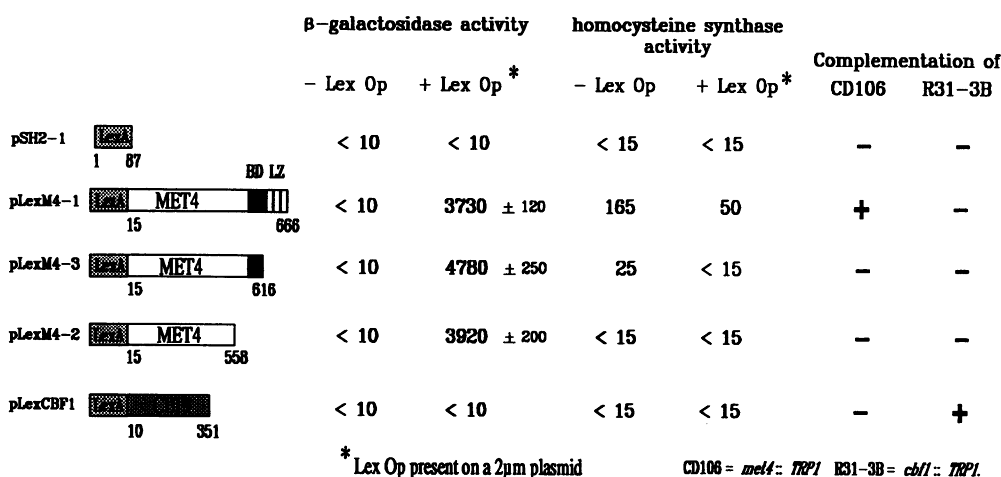

FIG. 5. Activation by the LexA-MET4 and LexA-CBF1 fusion proteins in a *met4*-disrupted strain. The strain used is CD106 (*met4*::*TRP1*). Transformants were grown in synthetic medium containing 0.05 mM L-methionine (derepressing condition) but lacking histidine and uracil to maintain selection for the multicopy plasmids. The target plasmids are pLR1Δ1 (lacking the *lexA* operator [44]) and pSH18-34 (16a), which contains six overlapping *lexA* operators inserted in the *Xho*I site of pLR1Δ1. Homocysteine synthase is expressed from the wild-type chromosomal *MET25* gene. Values are averages of assays of three transformants. One unit of activity is equal to 1 nmol of substrate transformed per min per mg of protein. In addition, the ability of each plasmid to complement the methionine auxotrophy of strain CD106 or R31-3B is indicated. Op, operator(s).

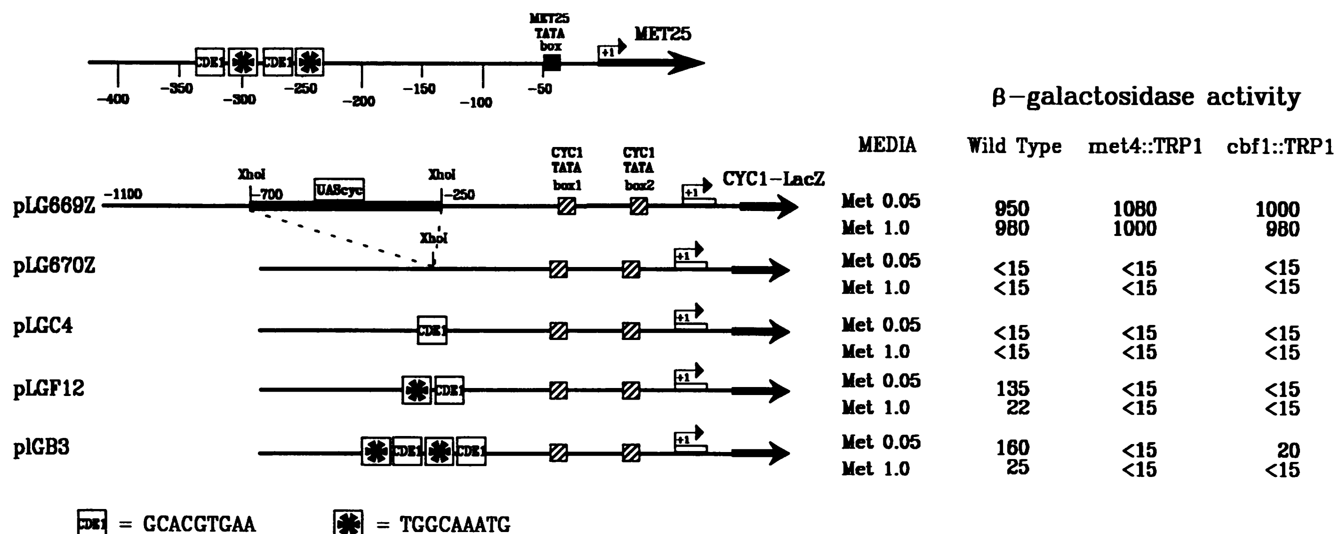

FIG. 6. β-Galactosidase activities expressed by the *MET25-CYC1-lacZ* fusion plasmids in different genetic backgrounds. Schematic representations of *MET25* regulatory region fusions to a *CYC1-lacZ* chimera are shown. Noted are the relative positions of the TATA boxes and starts of transcription. Plasmids pLGF12 and pLGB3 were constructed by inserting one and two copies of the 18-bp oligonucleotide, respectively, into the *Xho*I site of the pLG670Z (15). This 18-bp oligonucleotide includes nucleotides −311 to −294 of the *MET25* 5′ upstream region. As discussed in the text, the sequence 5′-TGGCAAATG-3′ is present in two copies up to the *MET25* gene (nucleotides −311 to −302 and −264 to −256). β-Galactosidase activities were measured in cell extracts from strains W303-1A (wild type), CD106 (*met4*::*TRP1*), and R31-3B (*cbf1*::*TRP1*) grown under derepressing (0.05 mM L-methionine) and repressing (1.0 mM L-methionine) conditions. All activities are means of three experiments performed with independent transformants. One unit of β-galactosidase activity is equal to 1 nmol of substrate transformed per min and per mg of protein.

when tested in a *met4* or *cbf1* null mutant under derepressing conditions.

DISCUSSION

Inactivation of the *CBF1* gene results in methionine auxotrophic yeast strains. This report clearly shows that this nutritional requirement is caused by the inability of these strains to assimilate sulfate. Northern blot experiments reveal that this defect is accounted for by an impairment in the transcriptional induction of certain of the genes governing the sulfate assimilation pathway. This result confirms our previous analysis that demonstrated the predominant role of the CDEI element in transcriptional activation of the *MET25* gene (40). However, despite the fact that CDEI elements are found in the 5′ upstream regions of all *met* genes, their full induction does not necessarily require the integrity of the CBF1 protein. Indeed, inactivation of the *cbf1* gene lowers the transcription of *MET16* to a nearly undetectable level, while it decreases that of *MET25* only about threefold and does not affect that of *MET3*. Although not noted by Mellor et al. (29), a similar decrease of *MET25* transcription in a *cbf1* null mutant is also revealed by their Northern experiments (Fig. 2A, lanes 9 and 12, in reference 29). In addition to the lack of PAPS reductase activity, the methionine auxotrophy of a *cbf1* null mutant also results from its incapacity to concentrate sulfate from the medium.

In contrast to the situation encountered in a *cbf1* null mutation background, in a *met4* background, all of the enzymatic activities required for the reductive assimilation of sulfate are lowered to nearly undetectable levels. Northern blot experiments show that the *MET4* product is required in *trans* for the proper induction of transcription of the corresponding genes. Indeed, neither *MET16* nor *MET25* transcripts were found in the RNA extracted from a *met4* strain grown under derepressing conditions. In addition, the role of MET4 appears to be limited to this pathway, since a *met4* mutant grows on all organic sulfur sources. The *MET4* gene was cloned, and its sequence reveals that it encodes a protein related to the bZIP proteins. However, the leucine zipper present at the C terminus of MET4 is preceded by a basic domain that does not resemble those of the bZIP activators previously described. Indeed, the basic domain of MET4 is immediately adjacent to the leucine zipper and not divided into two clusters.

Evidence that *MET4* encodes a transcriptional activator is provided by the use of LexA-MET4 fusion proteins. The fusion proteins bearing at least MET4 amino acids 15 to 558, fused to the DNA-binding domain of the bacterial repressor *lexA*, stimulate transcription from a *lexA* operator positioned upstream to the *GAL1* gene. The target gene is induced by hybrid proteins containing or lacking the C terminus of MET4. In contrast, induction of the *MET25* gene by these hybrids requires that they carry the C terminus of MET4 comprising the basic domain and leucine zipper. Taken together, these results suggest that the bZIP domain of MET4 is responsible for its DNA recognition either by binding directly to the DNA or through its interaction with another DNA-bound protein factor. Whether MET4 recognizes DNA by itself remains to be established. If not, the DNA-bound factor that would bring MET4 to the DNA cannot be the CBF1 protein, since a *MET25-CYC1-lacZ* gene fusion containing a CDEI element alone fails to express any activity. Other evidence is also provided by the residual transcription of the *MET25* gene in a *cbf1* null mutant.

The *MET25-CYC1-lacZ* gene fusion experiments reveal

that an 18-bp segment of the *MET25* promoter is capable of functioning as an upstream activation element. Furthermore, this element is capable of conferring regulation in response to the increase in intracellular AdoMet concentration. This 18-bp element contains a CDEI element and its preceding 9 bp in the *MET25* promoter. This 9-bp sequence (5'-TGG CAAATG-3') is repeated twice in the *MET25* 5' upstream region. It must be noted that a deletion of the region containing one repeat but not the CDEI element was previously shown to result in twofold decrease in the *MET25* transcription level (Fig. 2, deletion E, in reference 40). Moreover, the functioning of this 18-bp element depends on *MET4*, since the *MET25-CYC1-lacZ* gene fusion is not expressed in a *met4*-disrupted strain. This finding strongly suggests that MET4 recognizes, directly or indirectly, a motif contained within the sequence 5'-TGGCAAATG-3'. However, analysis of the 5' upstream region of the *MET* genes, the expression of which depends on MET4, has not allowed us to define a discernible consensus sequence. This lack of consensus perhaps reflects the fact that genes that require CBF1 may not bind the MET4 protein as well as do those that do not require CBF1.

The transcriptional activation of inorganic sulfur metabolism thus appears to require at least two *trans*-acting proteins, MET4 and CBF1. What might be the functional role of the CBF1 protein? The inability of the LexA-CBF1 fusion protein to stimulate the transcription through a *lexA* operator as well as the inability of CDEI to function alone as a upstream activation element strongly suggest that CBF1 does not operate by interacting with the components of the transcription apparatus. It was reported that a micrococcal nuclease-sensitive chromatin structure is lost on the *MET25* promoter in the absence of CBF1 (28). Here we show that the integrity of CBF1 is required for proper transcriptional activation of the *MET16* and *MET25* genes. Furthermore, induction of the *MET25-CYC1-lacZ* gene fusions also depends on CBF1. All of these results support the hypothesis that the binding of CBF1 to its cognate sequence CDEI contributes to the increased ability of MET4 to function as a transcriptional activator. CBF1 thus appears to be related to the family of yeast multifunctional DNA-binding proteins comprising GRF1 (RAP1), GRF2, and ABF1 (8, 12). A striking feature shared by these yeast DNA-binding proteins is the widespread occurrence of their binding sites in the yeast genome. These binding sites are furthermore found associated with both promoter elements and structures specialized in DNA transactions, such as centromeres, telomeres, and replication origins. Results of micrococcal nuclease experiments suggest that the function of these proteins is to increase the accessibility of other DNA-binding factors to their respective binding sites, perhaps by the exclusion of nearby nucleosome binding (23). Such a mechanism probably depends on the structural features of each promoter region. For example, the presence of poly(A) or poly(T) tracts was shown to prevent nucleosome formation in vitro (23). Such structural differences could explain why inactivation of the *CBF1* gene does not affect in the same manner the recognition by MET4 of each promoter region of the genes governing inorganic sulfur metabolism. In this regard, it must be noted that a long poly$(AT)_{19}$ tract is positioned near the TATA box of the *MET3* gene, the transcription of which depends on the integrity of MET4 but not on that of CBF1. A different conclusion about CBF1 function was drawn by Mellor et al. (29), who reported that a mutant form of CBF1, unable to bind in vitro to the *MET25* promoter, is still capable of maintaining methionine prototrophy. Therefore, they conclude that the CBF1 protein is able to function as a non-CDEI DNA-bound form in sulfur metabolism. However, this result could also reflect the sensitivity limits of the in vitro DNA-binding experiments compared with the situation existing in vivo.

Enzymatic analysis of a *cbf1* null mutant also revealed that this strain is defective in sulfate permeation. The loss of sulfate permease activity is a property shared by a number of *S. cerevisiae* mutants affected in the sulfate assimilation pathway. In particular, disruption of the chromosomal gene *MET16* (encoding PAPS reductase) results in a strain devoid of both PAPS reductase and sulfate permease activities. This phenotype is reversed by the presence of a *MET16* plasmid gene (41a). Such a concomitant loss of activities is also observed in *E. coli* but arises from a different mechanism. Indeed, *E. coli* cells constructed to lack both thioredoxin and glutaredoxin (essential cofactors of PAPS reductase) are not viable unless they also acquire an additional mutation in *cysA*, the structural gene for sulfate permease (34). These reversible (*S. cerevisiae*) or irreversible (*E. coli*) inactivations of sulfate permease are hypothesized to allow the survival of cells by protecting against the toxicity of inorganic phosphosulfur compounds (especially against that of PAPS). Although we cannot rule out the possibility that inactivation of *CBF1* abolishes transcription of the sulfate permease gene, the defect in sulfate permeation exhibited by a *cbf1*-disrupted strain may rather be caused by such a protective inhibition of the sulfate permease made necessary by the improper induction of the *MET16* gene.

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