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Role of the *Saccharomyces cerevisiae* General Regulatory Factor CP1 in Methionine Biosynthetic Gene Transcription

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***Saccharomyces cerevisiae* general regulatory factor CP1 (encoded by the gene *CEP1*) is required for optimal chromosome segregation and methionine prototrophy. *MET16-CYC1-lacZ* reporter constructs were used to show that *MET16* 5′-flanking DNA contains a CP1-dependent upstream activation sequence (UAS). Activity of the UAS required an intact CP1-binding site, and the effects of *cis*-acting mutations on CP1 binding and UAS activity correlated. In most respects, *MET16-CYC1-lacZ* reporter gene expression mirrored that of chromosomal *MET16*; however, the endogenous gene was found to be activated in response to amino acid starvation (general control). The latter mechanism was both GCN4 and CP1 dependent. *MET25* was also found to be activated by GCN4, albeit weakly. More importantly, *MET25* transcription was strongly CP1 dependent in *gcn4* backgrounds. The modulation of *MET* gene expression by GCN4 can explain discrepancies in the literature regarding CP1 dependence of *MET* gene transcription. Lastly, micrococcal nuclease digestion and indirect end labeling were used to analyze the chromatin structure of the *MET16* locus in wild-type and *cep1* cells. The results indicated that CP1 plays no major role in configuring chromatin structure in this region, although localized CP1-specific differences in nuclease sensitivity were detected.**

General regulatory factors form a family of sequence-specific DNA-binding proteins of *Saccharomyces cerevisiae*. Although structurally dissimilar, all of these factors are moderately abundant proteins with many genomic sites of interaction and are involved in diverse genetic processes. Members of the family include CP1, RAP1, REB1, and ABF1; with the exception of CP1, all are essential proteins. General regulatory factors have been shown to be associated with chromosome origins of replication (ABF1), telomeres (RAP1), and centromeres (CP1). In addition to their association with loci involved in chromosome maintenance, all have been implicated to some degree as positive and negative regulators of transcription (6, 7, 9). CP1 was the first member of the general regulatory factor family to be identified (5) and was originally characterized as an abundant protein, present in yeast extracts, that bound a conserved element of yeast centromeres referred to as centromere DNA element I (CDEI). Cloning and sequencing of *CEP1*, the gene encoding CP1, revealed the presence of a basic region-helix-loop-helix domain, a DNA-binding and dimerization motif common to a family of proteins involved in transcriptional regulation (8, 26). The analysis of mutants in which the *CEP1* gene had been disrupted confirmed the proposed role of CP1 in chromosome segregation and provided the first evidence that it was involved in other processes as well. Strains lacking a functional *CEP1* gene product were found to display an array of phenotypes including an increase in the frequency of mitotic and meiotic chromosome missegregation, slow growth, and a requirement for methionine (3, 8, 23, 26).

The physiological cause of *cep1* methionine auxotrophy is explained by the observed lack of 3′-phosphoadenylylsulfate reductase (40), the product of the *MET16* gene (38). The methionine biosynthetic (*MET*) genes are regulated in response to methionine availability (10, 29, 38, 39). In the presence of methionine, transcription is repressed via a mechanism that monitors the intracellular level of *S*-adenosylmethionine (AdoMet) (39). When the level of extracellular methionine is low, AdoMet repression is relieved and *MET* gene transcription is activated by a mechanism involving the positive *trans*-acting factor encoded by *MET4* (28, 40). Consensus CDEI sites are found upstream of almost all of the *MET* genes, and in the case of *MET25* (encoding *O*-acetylhomoserine sulfhydrylase), these CDEI sites are essential for normal transcriptional activation (39). The *cep1* methionine auxotrophy is suppressed by constitutive activation of PHO4, a basic region-helix-loop-helix transcription factor having a DNA-binding specificity very similar to that of CP1 (32). Presumably, PHO4 restores expression to CP1-dependent *MET* genes by binding to the upstream CDEI sites and reactivating transcription. That *cep1* methionine auxotrophy is suppressed by a known transcription activator implies that CP1 acts positively to activate *MET* gene expression.

Direct analysis of *MET* gene transcription in *cep1* mutants has yielded conflicting results. The steady-state mRNA levels of *MET2*, *MET3*, and *SAM2*, all of which possess upstream CDEI sites, appear normal in *cep1* strains (our unpublished observations). Mellor and coworkers reported no effect of the *cep1* (*cpf1*) mutation on either *MET25* or *MET16* expression (21, 27), while Thomas et al. (40) detected no *MET16* RNA and a threefold reduction in *MET25* RNA in a *cep1* strain. Where observed, the lack of normal *MET16* RNA in *cep1* mutants is presumably due to a defect in transcription; however, it is unclear why this member of the coregulated *MET* gene family should be so distinctly CP1 dependent or why such variable results are obtained. CP1 does not appear to be a typical transactivator protein (like PHO4, GAL4, and GCN4), since it lacks an autonomous transcription activation domain.

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TABLE 1. Yeast strains used in this study

Strain	Genotype
K23-9A	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200*
K23-9B	*MATα leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1*::*TRP1*
K23-9D	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 cep1*::*TRP1 pho80*::*LEU2*
K55-M47	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 met16-47*
K56-M48	*MAT***a** *leu2Δ1 lys2-801 trp1Δ1 ura3-52 ade2-101 his3Δ200 met16-48*
CD107	*MAT***a** *leu2 his3 ura3 ade2 trp1 met4*::*TRP1*
F113	*MAT***a** *ino1 ura3-52*
F212	*MAT***a** *ino1 ura3-52 gcn4-Δ1*
K64-T1	*MAT***a** *ino1 ura3-52 cep1*::*URA3*
K63-T1	*MAT***a** *ino1 ura3-52 gcn4-Δ1 cep1*::*URA3*

LexA-CP1 or GAL4 DNA-binding domain–CP1 fusion proteins fail to activate LexA or GAL4 DNA-binding site-driven reporter genes, respectively (reference 40 and unpublished observations). Furthermore, CDEI sites do not exhibit upstream activation sequence (UAS) activity when placed alone upstream of a reporter gene (7, 40). However, a CDEI, in combination with a second repeated element from the *MET25* promoter region, forms a methionine-responsive UAS that depends on both CP1 and *MET4* (40). The results obtained with this minimal *MET25* UAS suggest that CP1 provides an "accessory" function, the loss of which can be partially compensated for in the context of the native promoter. It is possible that CP1 is involved in recruiting other factors (such as MET4) to *MET* promoters, similar to the role proposed for RAP1 in *HIS4* expression (11). Like CP1, RAP1 activates transcription efficiently only in combination with other factors (7). Another possibility is that CP1 orders the nucleosomal structure of *MET* genes. A precedent for this is general regulatory factor REB1 (also known as factor Y), known to be a strong positioner of nucleosomes (13).

To better define the role of CP1 in the expression of *MET* genes, we have undertaken a systematic study of *MET16* expression in wild-type and *cep1* cells. We find that *MET16* transcription can be activated through two largely independent mechanisms, one triggered by methionine starvation and requiring MET4 (pathway-specific control), and the other triggered by starvation for many different amino acids and requiring GCN4 (general control). CP1 is required for activation by either pathway and mediates its function through the CDEI site in the *MET16* promoter. In addition, we have analyzed the chromatin structure of the *MET16* promoter in wild-type and *cep1* strains and have found differences in the region around the CDEI site, consistent with this region being more accessible in the wild-type strain. These results suggest that CP1 acts directly at *MET* gene promoters and is essential for the normal pathway-specific activation of *MET* gene transcription.

MATERIALS AND METHODS

Strains, media, and general methods. Yeast strains used in this study are listed in Table 1. Strains F113 and F212 were obtained from A. Hinnebusch; all others were constructed by standard genetic methods. Strains K64-T1 and K63-T1 were created by disrupting the *CEP1* gene in strains F113 and F212, respectively (23). Strains K23-9A, K23-9B, and K23-9D are meiotic segregants of diploid K23 (32). Media were as described previously (3). Yeast transformations were performed by the lithium acetate procedure (20) as modified by Schiestl and Gietz (36). DNA sequencing was carried out with the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Inc.) as instructed by the manufacturer, and β-galactosidase activity was assayed in whole yeast cells as described previously (34).

***CYC1-lacZ* reporter plasmids.** *CYC1-lacZ* reporter plasmids were derived from plasmid pLG669Z, obtained from L. Guarente (15). pLG669Z was cut with *Xho*I to remove the *CYC1* UAS (yielding a linearized vector equivalent to Guarente's pLG670Z) and, after the ends were blunted with Klenow polymerase, ligated with the 305-bp *Rsa*I fragment spanning positions 411 to 715 of the *MET16* promoter region (38). Plasmids pRB137-2 and pRB137-4 contain a single insert in opposite orientations. Plasmid pRB137-1 lacks an insert and served as the UAS-less control. Site-directed mutagenesis of the *MET16* CDEI site was performed with the Altered Sites *In Vitro* Mutagenesis System (Promega Corp.) as specified by the manufacturer. For mutagenesis, the 305-bp *Rsa*I *MET16* fragment described above was inserted into the phagemid pALTER-1. The mutagenic oligonucleotides were as follows: M16-47, 5′-TCATCATTTCACTAGTG GCTAGTAAAAGAA-3′ (*Spe*I site); M16-33, 5′-TTTTTATTTTTATCACTAG TCACGTGGCTA-3′ (*Spe*I site); M16-39, 5′-TCATCATTTCACATGGCTAG TAAAAGAA-3′; and M16-48, 5′-TCATCATTTCAGCTGGCTAGTAAAAG AA-3′ (*Pvu*II site). Reporter plasmids carrying these mutated elements were constructed by removing each insert from the mutagenesis vector as an *Rsa*I-*Sal*I fragment and cloning it between the *Sma*I and *Xho*I sites of pLG669Z. The latter series of *CYC1-lacZ* reporters (the pKM11 series) lack, in addition to the *CYC1* UAS, all *CYC1* sequences upstream of the *Xho*I site. The site-directed mutations were confirmed by DNA sequencing of the inserts contained in the cognate *CYC1-lacZ* plasmids.

Pop-in/pop-out mutagenesis of *MET16*. The chromosomal *MET16* promoter mutations in strains K55-M47 and K56-M48 were generated by pop-in/pop-out mutagenesis (35). The plasmid used to construct the replacement vectors (pKM46) contained *MET16* sequences from position 1 to the *Rsa*I site at position 412 joined by a *Sma*I-*Bam*HI linker (CCCGGGATCC) to sequences from position 708 (*Acc*I site) to position 964 (*Cla*I) and cloned between the *Xba*I and *Cla*I sites of the *URA3* integrating plasmid pRS306 (37). Mutated *MET16* promoter fragments were excised from the corresponding pALTER-1 phagemids as *Rsa*I-*Bam*HI fragments and inserted at the *Sma*I-*Bam*HI linker of pKM46. The resulting plasmids, pKM47 (*met16-47*) and pKM48 (*met16-48*), were used for pop-in/pop-out mutagenesis. For the pop-in, pKM47 and pKM48 were cut with *Bam*HI and used to transform strain K23-9A to uracil prototrophy. Transformants which had correctly integrated the plasmids were identified by Southern blotting. For each mutation, one integrant was chosen and derivatives which had excised the plasmid (pop-outs) were selected with 5-fluoroorotic acid (4). Retention of the mutations in the genome was confirmed by Southern blotting (both mutations create new restriction sites in the *MET16* 5′-flanking DNA). Sequences surrounding the *Acc*I site at position 708, mutated by insertion of the *Sma*I-*Bam*HI linker in the replacement vectors, are restored during the pop-in recombination event.

Northern (RNA) blotting. Procedures for isolating total cellular RNA (16) and blot hybridization (1) were as described previously. For the nutritional shift experiments, cells were pregrown in synthetic complete medium supplemented with 1 mM methionine ($SCM_{1.0}$), harvested by centrifugation, washed in the same medium lacking methionine (SCM_0) and resuspended in a small volume of SCM_0. This cell suspension was used to inoculate equal volumes of SCM_0 and $SCM_{1.0}$ to give initial culture densities of 0.5×10^7 to 1.0×10^7 cells per ml. Samples were collected periodically following the nutritional shift and used to isolate total RNA. To analyze GCN4-dependent expression, cells were pregrown for several hours in SCM lacking all aromatic amino acids before being starved for tryptophan by adjusting the cultures to 1 mM 5-methyltryptophan (5-MT). Growth was allowed to continue, and samples were removed periodically and used to isolate total RNA.

Probes used to detect various mRNAs were labeled with [α-^{32}P]dCTP by using a random primed DNA-labeling kit (Boehringer Mannheim) as specified by the manufacturer. The following gel-purified fragments were used as probes: for *MET16*, the 563-bp *Cla*I-*Eco*RI fragment (38); for *MET25*, the 271-bp *Eco*RI-*Xba*I fragment (22); for *ACT1*, the 300-bp *Bgl*II-*Dra*I fragment (30); and for *HIS4*, the *Sac*I-*Sph*I fragment (12). All blots were quantitated with a Betagen Betascope 603 Blot Analyzer.

DNA-binding assays. The relative apparent binding constants for CP1 binding to the mutated CDEI sites were measured by a competition gel electrophoretic mobility shift binding assay (2). Test probes were synthesized by PCR with, as template, pALTER-1 plasmids containing wild-type and mutated *MET16* promoter segments and primers flanking the polylinker region (puceco, 5′-GAAT TCGAGCTCGGTACCC-3′; puchind, 5′-AAGCTTGCATGCCTGCAGGTC-3′). A reference DNA probe was synthesized by PCR with the same primer set and with pDR1-7 as the template. Plasmid pDR1-7 contains the CDEI site of *CEN3* cloned into the *Bam*HI site of pUC18 (24). One primer (puceco) was end labeled with [γ-^{32}P]ATP by using polynucleotide kinase to a predicted specific activity of ~1,000 Ci/mmol, and the same preparation of labeled primer was used in each amplification reaction to generate probes of equal specific radioactivity. The resulting test and reference probes, 362 and 82 bp in length, respectively, were purified by polyacrylamide gel electrophoresis and isolated by electroelution. DNA-binding reactions were carried out exactly as described previously (24) with purified yeast CP1 (2). Test and reference probes were added to the reaction mixtures in various ratios. After electrophoresis, the gels were dried and all four species of DNA (bound and free test DNA, bound and free reference

DNA) were quantitated simultaneously with the Betagen Betascope 603. Relative apparent equilibrium constants were calculated from the following equation (2):

$$K_{rel} = \frac{K_{test}}{K_{ref}} = \frac{T_b}{R_b} \cdot \frac{R_f}{T_f}$$

where K_{rel} is the relative apparent equilibrium constant, K_{test} and K_{ref} are the individual equilibrium constants for CP1 binding to test and reference probes, respectively, T_b and R_b are the counts per minute of CP1-bound test and reference probes, respectively, and T_f and R_f are the counts per minute of unbound (free) test and reference probes, respectively. The measured K_{rel} for the wild-type *MET16* CDEI site versus the *CEN3* CDEI site was 0.65 ± 0.08 (mean ± standard error of the mean for 12 separate determinations). The average variation from the mean in independent determinations of K_{rel} values for the four mutant CDEIs was 40%. This method for measuring relative binding constants is general and requires only that test and reference DNAs differ sufficiently in electrophoretic mobility such that both bound and free forms of each are resolvable and that both probes can be synthesized with at least one common PCR primer.

Analysis of chromatin structure. The chromatin structure of the *MET16* promoter was analyzed essentially as described by Hull et al. (19). Cultures were prepared exactly as described for the Northern blot experiments (see above) and harvested at a density of approximately 10^7 cells per ml (2 to 3 h after the nutritional shift). The cells were collected by centrifugation and suspended in 15 ml of 40 mM EDTA. After the addition of 100 μl of 2-mercaptoethanol, the samples were left at room temperature for 5 min. The cells were again collected by centrifugation and suspended in either $SCM_{1.0}$ or SCM_0, corresponding to their growth medium, containing 1 M sorbitol. Zymolyase-100T (ICN) was added to 1.25 mg/ml, and the cells were incubated for 30 min at 30°C with intermittent agitation. Cell wall digestion was stopped by addition of ice-cold hypotonic buffer (100 mM NaCl, 6 mM Tris-HCl [pH 7.4], 6 mM $MgCl_2$) containing 1 mM $CaCl_2$. The spheroplasts were pelleted by centrifugation, resuspended to 100 cell units/ml (cell units = optical density at 600 nm × volume of original culture in milliliters) in hypotonic buffer containing 1 mM $CaCl_2$, 0.05% Triton X-100, 2 μg of pepstatin per ml, and 1 mM phenylmethylsulfonyl fluoride, transferred to prechilled 7-ml Dounce homogenizers, and broken by six or seven strokes with the loose-fitting pestle. Aliquots, dispensed to microcentrifuge tubes on ice, were prewarmed at 30°C for 3 min before addition of micrococcal nuclease (MNase; Sigma Chemical Co.) to the final concentrations specified in the legend to Fig. 6. Digestion was allowed to proceed for 3 min and then terminated with an equal volume of stop solution (1 M NaCl, 50 mM Tris-Cl [pH 7.9], 2% sodium dodecyl sulfate, 50 mM EDTA). Genomic DNA was isolated as described previously (19), digested to completion with *Eco*RI and *Xba*I, and separated by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and analyzed by indirect end labeling (42), with the same *Eco*RI-*Cla*I *MET16* probe as was used for Northern analysis. In most experiments, indirect end labeling was also carried out with a probe complementary to the region adjacent to the upstream *Xba*I site. In all cases, the pattern of MNase cleavage sites was similar to that obtained with the *Eco*RI-adjacent probe. Before electrophoresis, the DNA samples were spiked with 10 ng of lambda *Hin*dIII-*Eco*RI molecular weight markers. After the blot was probed for *MET16* DNA, the membrane was stripped and reprobed with labeled lambda DNA to detect the molecular weight standards. For each lane, a standard curve was generated and used to determine the size of the *MET16* fragments visualized in the same lane. The standard deviation of the deduced MNase cleavage sites, within and between experiments, ranged from 6 to 31 bp. Densitometric scans of blot autoradiograms were obtained with a Biomed Instruments soft laser densitometer (model SLR-1D/2D).

RESULTS

The 5′-flanking region of the *MET16* gene bears a CP1-dependent UAS. As a first step toward identifying *cis*-acting elements important for CP1-dependent regulation of *MET16*, we constructed a reporter gene containing 305 bp of *MET16* 5′-flanking DNA in place of the UAS element of the *CYC1-lacZ* reporter plasmid pLG669Z (15). The position of the single CDEI site within this fragment and the sequences immediately surrounding it are shown in Fig. 1. Yeast strains carrying various reporter plasmids (Fig. 1) were assayed for β-galactosidase activity following growth under conditions selective for the plasmid and either repressive (1 mM methionine) or nonrepressive (0.05 mM methionine) for *MET* gene expression. In the absence of its UAS element, the *CYC1-lacZ* reporter was expressed at a low level that varied neither with respect to the genetic background of the host strain nor with the methionine concentration of the medium (Table 2, plasmid pRB137-1). In contrast, when the *MET16* upstream region

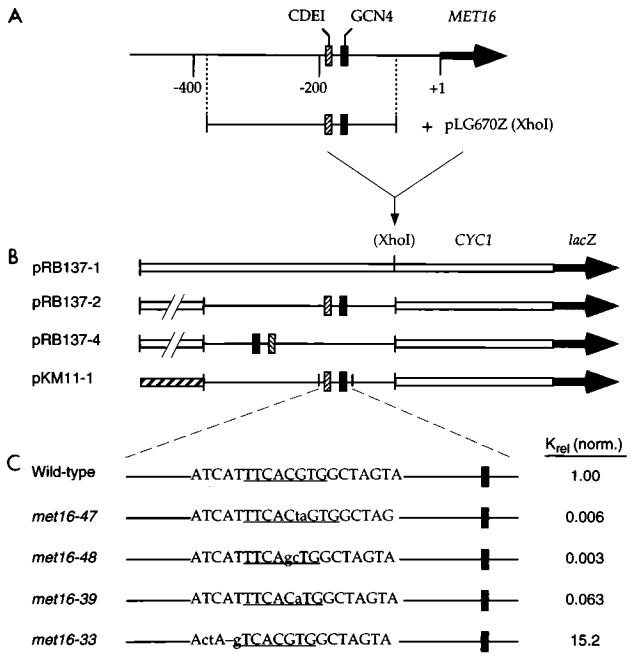

FIG. 1. Structure of *MET16-CYC1-lacZ* reporter genes. (A) Schematic of the *MET16* locus with the coding region depicted as a solid arrow. The boxes (not to scale) represent the binding sites for CP1 (cross-hatched) and GCN4 (solid), and the numbers indicate distance, in base pairs, relative to the translation initiation codon. To construct the reporter plasmids, a 305-bp *Rsa*I fragment from the *MET16* 5′-flanking DNA was inserted into the Klenow-blunted *Xho*I site of plasmid pLG670Z (derived by *Xho*I digestion of pLG669Z) (15). (B) *CYC1-lacZ* reporter plasmids showing the presence and orientation of the *MET16 Rsa*I fragment within each construct. Plasmid pKM11-1 is essentially the same as pBR137-2, except that it lacks *CYC1* sequences upstream of the *Xho*I site. Deletion of this segment places the *URA3* gene immediately upstream of the *MET16* insert. (C) Site-directed mutations were made within the CDEI site of the *MET16 Rsa*I fragment, and the mutated *MET16* elements were inserted in place of the wild-type element in pKM11-1. The specific sequence alterations are shown with the 8-bp CDEI consensus underlined. Lowercase letters denote changes from the wild-type sequence, and the hyphen indicates the additional 1-bp deletion in *met16-33*. The relative affinities of CP1 for the altered elements (K_{rel}s) were determined by an in vitro DNA-binding assay (see Materials and Methods) and are expressed relative to the wild-type sequence.

replaced the *CYC1* UAS (pRB137-2), β-galactosidase activity in the wild-type strain was regulated in response to methionine. In its normal orientation relative to the initiation site, the element directed a >100-fold increase in activity under the nonrepressive conditions (50 μM methionine); thus, this 305 bp of DNA contained all the information sufficient to confer methionine-responsive expression to the reporter gene. Expression of the *MET16* reporter depended on CP1. When *cep1* strains were grown under nonrepressing conditions, the level of β-galactosidase activity was reduced 100-fold (Table 2). The low observed activity was not due to loss or mutation of the reporter plasmid, since full activity could be restored to reporter-carrying *cep1* strains by transformation with a plasmid carrying *CEP1* (data not shown).

The methionine auxotrophy of *cep1* strains is suppressed by mutations which cause constitutive activation of PHO4, a transactivator of genes involved phosphate metabolism (32). Because CP1 and PHO4 are structurally similar and possess nearly identical DNA-binding specificities, it is presumed that PHO4 suppressors act by binding to promoter CDEI sites and activating *MET* gene expression. We tested this hypothesis by measuring expression of the *MET16* reporter construct in a *cep1 pho80* double mutant. PHO80 is a negative regulator of

TABLE 2. β-Galactosidase activities of *MET16-CYC1-lacZ* reporter genes

Reporter	Met concn (mM)[a]	β-Galactosidase activity (*n*)[b]		
		Wild type	*cep1*	cep1 pho80
pRB137-1	0.05	0.58 ± 0.17 (5)	0.60 ± 0.19 (3)	0.94 ± 0.19 (2)
	1.0	0.47 ± 0.13 (4)	0.58 ± 0.15 (3)	0.40 ± 0.04 (2)
pRB137-2	0.05	35.3 ± 8.4 (6)	0.37 ± 0.04 (3)	3.9 ± 3.2 (9)
	1.0	0.18 ± 0.12 (5)	0.13 ± 0.02 (3)	0.22 ± 0.08 (7)
pRB137-4	0.05	6.1 ± 2.4 (3)	0.57 ± 0.13 (3)	3.8 ± 2.5 (9)
	1.0	0.43 ± 0.13 (4)	0.56 ± 0.13 (3)	0.66 ± 0.07 (6)
pKM11	0.05	87.7 ± 25 (23)	0.17 ± 0.03 (3)	0.76 ± 0.06 (2)
	1.0	0.19 ± 0.16 (20)	0.11 ± 0.05 (4)	0.19 ± 0.04 (2)

[a] In SCM lacking uracil.
[b] Cultures were grown to an optical density at 600 nm of ≥2.5 before β-galactosidase activity was measured in whole cells. β-Galactosidase activities in Miller units are reported as the mean ± standard deviation with the number of determinations in parentheses. Host strains: wild type, K23-9A; *cep1*, K23-9B; *cep1 pho80*, K23-9D.

PHO4, and *pho80* mutations lead to constitutive activation of PHO4. β-Galactosidase activity was partially restored in the *cep1 pho80* double mutant (Table 2). When present in the suppressor strain, the reporter expressed about 1/10 the level of β-galactosidase activity observed in the wild-type strain, consistent with the observation that *pho80* only partially suppresses the *cep1* methionine requirement (32). Surprisingly, PHO4-mediated activation was observed only under conditions of low extracellular methionine, even though PHO4 is constitutively active in this strain. Either PHO4-mediated activation requires at least one other factor that is activated by methionine starvation, or PHO4 is unable to overcome AdoMet-mediated repression. In any event, the reporter activity observed in the *cep1 pho80* strain supports the view that PHO4-mediated suppression of *cep1* methionine auxotrophy results from partial restoration of CP1-dependent gene transcription.

Plasmid pRB137-4 carries the *MET16* promoter fragment in the opposite orientation (Fig. 1). β-Galactosidase expression driven by the inverted element was still regulated in response to methionine availability, but in this orientation the promoter fragment conferred weaker UAS activity (Table 2). Compared with results for its native orientation, the inverted element was only 17% as effective in activating the reporter gene; however, activity was still completely dependent on CP1 and was restored in the *cep1 pho80* suppressor strain. Interestingly, in the double mutant, reporter activity was unaffected by the orientation of the *MET16* UAS (compare the activities of pRB137-2 and pRB137-4 in the *cep1 pho80* strain), suggesting that CP1- and PHO4-mediated activations are mechanistically different. It is likely that PHO4 does not simply substitute for CP1 but instead bypasses the normal CP1-mediated activation mechanism.

The foregoing results indicated that CP1 was required in *trans* for activity of the *MET16* UAS. To determine if the CP1 DNA binding site was required in *cis*, site-directed mutations were made within the single CDEI site, and the effects of those mutations on CP1 binding and UAS activity were assayed. The CDEI consensus sequence, RTCACRTG (where R is purine), has been defined in comparisons of yeast centromeric DNAs (14). The CDEIs in *MET* gene promoter regions differ slightly from that consensus in showing a strong preference for G at position 6, which occurs within the palindromic core (CAC GTG), and in lacking a marked preference for a purine at position 1. The *MET16* CDEI has a T at position 1 and a G at position 6 (Fig. 1). The mutation *met16-47* inserted the dinucleotide TA into the center of the CACGTG palindrome (creating an *Spe*I site), while *met16-48* inverted the central CG. Both of these mutations were expected to have strong negative effects on CP1 binding. The mutation *met16-33* created an *Spe*I site just upstream of the CDEI site, improving homology to consensus by changing position 1 to a G and, coincidentally, altering nucleotides outside of the CDEI. The mutation *met16-39* is a G-to-A transition at position 6, a change known to have no effect on centromere CDEI function (31).

TABLE 3. Effects of CDEI mutations on expression of the *MET16-CYC1-lacZ* reporter gene

Allele	β-Galactosidase activity (*n*)[a]	
	0.05 mM Met	1.0 mM Met
Wild type[b]	87.7 ± 25 (23)	0.19 ± 0.16 (20)
met16-47	0.21 ± 0.20 (3)	0.08 ± 0.06 (3)
met16-48	0.16 ± 0.05 (7)	0.10 ± 0.03 (7)
met16-39	12.7 ± 5.5 (8)	0.13 ± 0.04 (8)
met16-33	16.4 ± 2.0 (5)	0.18 ± 0.17 (5)

[a] As in Table 2; the host strain was K23-9A.
[b] From Table 2.

Binding of purified yeast CP1 to the mutated *MET16* promoter fragments was analyzed in quantitative electrophoretic mobility shift assays. The assays measured the equilibrium constant of CP1 binding to the *MET16* DNA probe relative to a reference probe containing the CDEI site of *CEN3* (see Materials and Methods). CP1 binding to the wild-type *MET16* UAS was reduced 1.5-fold relative to *CEN3* (a measured K_{rel} of 0.67). K_{rel} values for the *MET16* CDEI mutants, normalized to wild-type *MET16*, are given in Fig. 1. As expected, the *met16-47* and *met16-48* mutations reduced CP1 binding significantly (170- and 300-fold, respectively). Changing the G at position 6 to A (*met16-39*), a nucleotide found in 6 of 15 centromeric CDEI sites, lowered the binding constant 16-fold. The *met16-33* UAS had a 15-fold-increased K_{rel}, confirming the importance of position 1 in CP1 recognition (2).

CYC1-lacZ reporter constructs containing the mutated *MET16* UAS fragments were tested for β-galactosidase expression. This series of plasmids (pKM11 series) differed from the pRB137 series in that the vector lacked *CYC1* sequences upstream of the *Xho*I cloning site. Removal of this DNA led to a 2.5-fold increase in β-galactosidase activity, but, as before, expression was completely dependent on CP1 (Table 2). All CDEI site mutations affected the levels of β-galactosidase induced in the presence of 50 μM methionine (Table 3). The *met16-47* and *met16-48* mutations resulted in complete loss of activity, while the *met16-39* UAS drove β-galactosidase expression to only 14% of the wild-type level. The correlation between the affinity of these mutated promoter elements for CP1 and their ability to activate expression of the reporter gene in response to lowered extracellular methionine levels strongly suggests that CP1 acts through the CDEI site. In the case of *met16-33*, CP1 binding affinity did not correlate with UAS activity; binding was increased 15-fold, while β-galactosidase expression was reduced about 5-fold (Fig. 1; Table 3). Significantly, the *met16-33* mutation changed nucleotides both within and outside of the CDEI site. Analysis of the minimal UAS element derived from the *MET25* promoter has shown that a CDEI site and the 10 bp adjacent to it are essential (40). The results with the *met16-33* mutant may indicate that the same is true for *MET16*; i.e., a critical *cis*-acting element occurs immediately adjacent to the CDEI.

Regulated expression of the chromosomal *MET16* gene requires CP1 and an intact CDEI site. Analysis of the *MET16-CYC1-lacZ* reporter genes allowed us to define a region of the *MET16* 5′-flanking DNA containing the sequences necessary for methionine-responsive regulation and to identify the CDEI site as an essential *cis*-acting element. Next, we wanted to extend those observations to the *MET16* gene in its native context. Northern analysis was used to measure steady-state *MET16* mRNA levels during growth in the presence of methionine (basal expression) or at times following a shift into medium lacking methionine (derepressed expression). To control for loading and mRNA integrity, Northern blots were probed to detect the constitutively expressed *ACT1* (actin) mRNA. The *cep1* strains were found to contain lower steady-state levels of actin mRNA than did wild-type cells grown under similar conditions (see the legend to Fig. 2). To avoid a possible bias, the cumulative data quantitated in Fig. 2B have been expressed relative to total cellular RNA. In other cases, when fewer repetitions were performed (see Fig. 3 to 5), the data were standardized to the internal actin "control." In these other cases, *MET16* (and other individual) RNAs in the *cep1* strain may be systematically overestimated.

A representative blot is shown in Fig. 2A, and the quantitated data are shown in Fig. 2B. For reference, *MET25* RNA was also analyzed. Methionine withdrawal led to a coordinate increase in *MET16* and *MET25* RNA levels. Maximum *MET16* mRNA levels, which were reached within 2 h following the medium shift, were increased 10-fold over basal levels; thereafter, they slowly declined. The *cep1* mutant did not exhibit the large increase in the level of *MET16* mRNA upon methionine limitation; however, a low level of *MET16* transcript was detected. Basal and derepressed *MET16* mRNA levels were reduced 2.5- and 7-fold, respectively. The *cep1* transcription defect was specific to *MET16*, as *MET25* mRNA levels were reduced less than twofold in the mutant. Because of the longer generation time of *cep1* mutants, it was possible that the observed decrease in *MET16* expression in the *cep1* strain was attributable to altered kinetics of induction, but time course experiments revealed that from as early as 20 min following the medium shift to as late as 8 h, *cep1* strains never fully derepressed *MET16* (data not shown).

MET16 expression was also examined in the *cep1 pho80* suppressor strain. Figure 2A shows the typical result; *MET16* mRNA levels were consistently elevated in samples prepared from the *cep1 pho80* strain when grown under methionine limitation. In experiments in which *cep1* and *cep1 pho80* strains were directly compared, the *cep1 pho80* strain expressed, on average, 2.4-fold more *MET16* mRNA (measured at 4 h) than the *cep1* strain did. It is possible that *MET16* mRNA levels continue to rise in the suppressor strain, but experiments were terminated after 4 h because the *cep1* mutant ceases to divide shortly thereafter. Phenotypically, the 2.4-fold increase in the level of *MET16* mRNA in the *cep1 pho80* strain is probably significant (see below). As with the reporter gene, expression of the chromosomal *MET16* gene in the *cep1 pho80* strain was responsive to methionine availability. Thus, despite regulation by a gratuitous activator (PHO4), *MET16* retains its normal methionine-responsive expression pattern, probably as a result of normal AdoMet-mediated repression.

To determine if the CDEI mutations which abolished expression of the *MET16-CYC1-lacZ* gene also effected expression of the chromosomal *MET16* gene, the native copy of *MET16* was replaced with versions carrying the *met16-47* and *met16-48* promoter mutations. The resulting isogenic strains, differing only in sequence within the CDEI of the *MET16* promoter, were analyzed for their ability to express *MET16*

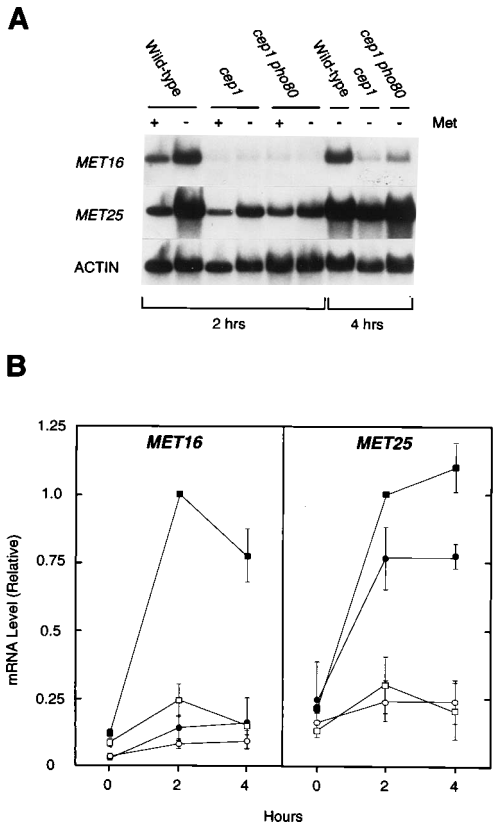

FIG. 2. Northern blot analysis of *MET16* RNA. Isogenic strains K23-9A (wild type), K23-9B (*cep1*), and K23-9D (*cep1 pho80*) were pregrown in 1.0 mM methionine-containing medium before being shifted into medium lacking methionine or, as the control, back into fresh methionine-containing medium. At the indicated times, samples were collected and the total RNA was extracted and analyzed by Northern blotting. *MET16*, *MET25*, and actin RNAs were detected sequentially after stripping the membrane. (A) A composite of radiographic images from a single experiment is shown to illustrate the typical result at 2- and 4-h time points in all three strains. (B) *MET16* and *MET25* mRNA levels in the wild-type and *cep1* strains were quantitated, combining data from eight experiments with an average of five independent determinations for each datum point. Values are expressed relative to total RNA and are normalized to the level of the corresponding transcript in the methionine-starved wild-type strain at the 2-h time point. Error bars indicate standard deviation. □, wild type + Met; ■, wild type − Met; ○, *cep1* + Met; ●, *cep1* − Met. The normalized values for actin RNA were (mean ± standard deviation with the number of determinations in parentheses): wild type + Met, 1.01 ± 0.19 (n = 23); wild-type − Met, 1.09 ± 0.18 (n = 25); *cep1* + Met, 0.81 ± 0.24 (n = 23); *cep1* − Met 0.74 ± 0.19 (n = 25).

and to grow in the absence of methionine. Both mutant strains were methionine bradytrophs; i.e., they grew poorly on synthetic medium lacking methionine. Whereas the wild-type strain formed 1- to 2-mm colonies after 3 days at 30°C, the *met16-48* strain required 5 days and the *met16-47* mutant required 1 week or more (data not shown). (The *cep1* mutant does not form even microcolonies after 7 days.) To analyze *MET16* expression, cells were shifted into medium lacking methionine and mRNA levels were measured as before. While both mutations reduced CP1 binding affinity more than 150-fold and completely abolished expression of the *MET16* reporter gene, neither completely inactivated expression of chromosomal *MET16* (Fig. 3; also see Fig. 5), consistent with the fact that neither mutation caused absolute methionine auxotrophy. The *met16-47* mutation was at least as severe in its effect on *MET16* expression as the *trans*-acting *cep1* mutation was, but, as would be predicted from growth phenotype, the

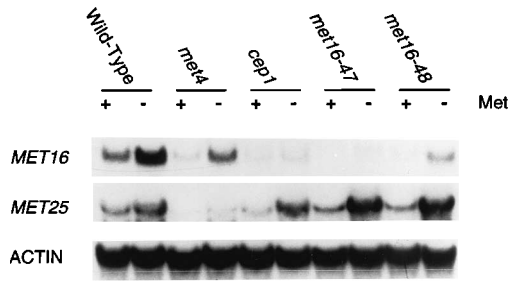

FIG. 3. Effect of *MET16* promoter mutations on *MET16* and *MET25* RNA levels. Strains K23-9A (wild type), K23-9B (*cep1*), K55-M47 (*met16-47*), K55-M48 (*met16-48*), and CD107 (*met4*) were shifted to medium lacking methionine. Total cellular RNA was extracted from samples collected 2 h following the shift and analyzed by Northern blotting. Quantitation is shown in Fig. 5.

met16-48 mutation had a less severe effect. The relatively small difference in *MET16* mRNA levels between the *met16-47* and *met16-48* strains translated into a measurable difference in their methionine-free growth rates, similar to the observation made for *cep1* and *cep1 pho80* suppressor strains. Interestingly, the *met16-47* and *met16-48* mutations appeared to increase the steady-state level of *MET25* mRNA (Fig. 3; also see Fig. 5). This response is probably due to general control (see below).

Transcription of *MET16* is regulated by a CP1-dependent general control response. Previous studies had indicated that the expression of *MET16* and *MET25* were absolutely dependent on the positive activator MET4 (40). Under the induction conditions used here, *MET25* transcription was observed to be MET4 dependent, but significant *MET16* transcription was observed in a *met4* mutant (Fig. 3). This led us to consider the existence of an additional control mechanism that might affect *MET16*. A distinguishing feature of the *MET16* 5′-flanking region is the presence of a consensus GCN4-binding site, an element involved in coordinating the expression of many amino acid biosynthetic genes in a response known as general control (18). To determine if *MET16* was regulated by GCN4 and, if so, what role CP1 might play in the response, the expression of *MET16* was analyzed under growth conditions known to trigger general control. Cells were pregrown in synthetic medium lacking all aromatic amino acids before being starved for tryptophan by the addition of 5-MT (11). RNA was prepared from samples taken at various times thereafter, and Northern analysis was performed. As shown in Fig. 4, *MET16* mRNA levels were rapidly elevated in response to tryptophan starvation. Within 1 h of addition of 5-MT, *MET16* mRNA was fully induced, and thereafter the levels declined. As a control, the blot was reprobed for transcripts of *HIS4*, a gene known to be regulated by general control (18). The pattern of *HIS4* expression was indistinguishable from that of *MET16* expression. Induction of both genes was due to the general control pathway, as demonstrated by the lack of induction of both *MET16* and *HIS4* in the *gcn4* mutant. The ability of 5-MT to induce *MET16* expression was reduced fourfold in the *cep1* mutant, while *HIS4* RNA levels were largely unaffected, indicating that CP1 was involved specifically in the general control response of *MET16*. The 5-MT response was intact in a *met4* mutant. Thus, *MET16* can be activated by general control independently of methionine-specific activation mediated by MET4. Significantly, both activation pathways appear to require CP1 for optimum activity.

The existence of the GCN4 activation pathway for *MET16* prompted us to reevaluate the CP1 dependence of *MET* gene expression in the absence of possible interference by GCN4.

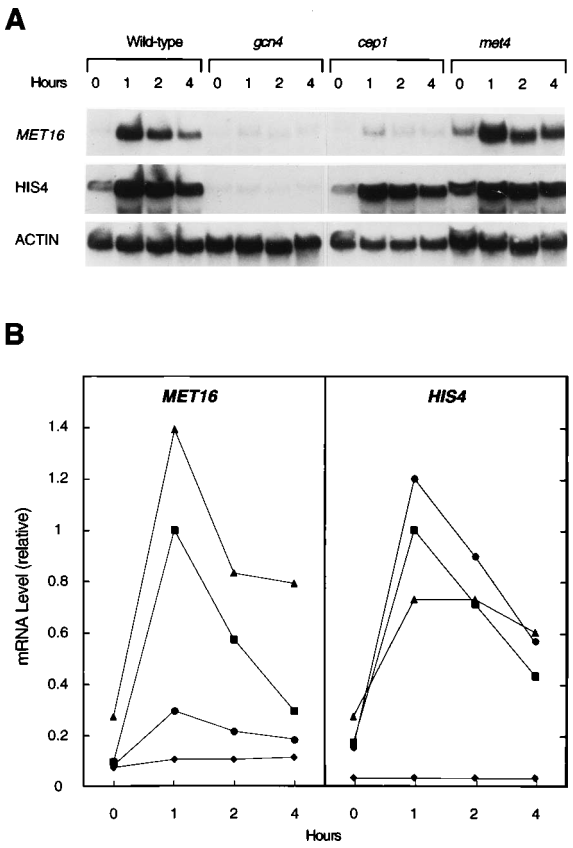

FIG. 4. General control of *MET16* expression. Strains F113 (wild type), F212 (*gcn4*), K23-9B (*cep1*), and CD107 (*met4*) were grown in SCM lacking aromatic amino acids. From each culture, an initial sample was removed and the remaining portion was adjusted to 1 mM 5-MT, after which additional samples were removed at the times indicated. Total cellular RNA was extracted from the samples and analyzed by Northern blotting. (A) Composite of radiographic images of the blot probed successively for *MET16*, *HIS4*, and actin RNA. (B) Relative mRNA level plotted against time for *MET16* and *HIS4* RNAs. The values were corrected for recovery of actin RNA and normalized to the level of the corresponding transcript in the wild-type strain at the 1-h time point. Symbols: ■, wild type; ◆, *gcn4*; ●, *cep1*; ▲, *met4*.

MET16, *MET25*, and *HIS4* RNA levels were analyzed in strains of various genetic backgrounds after derepression in methionine-free medium or induction by 5-MT (Fig. 5). The involvement of CP1 in both *MET16* activation pathways was confirmed by the disabled response of the *met16-47* and *met16-48* mutants to either induction condition. The lowest levels of *MET16* RNA were found in the *cep1 gcn4* double mutant. This implied that the low level of *MET16* expression observed in *cep1* mutants is due to GCN4. The pattern of *MET25* expression differed from that of *MET16* expression. In the wild-type background, *MET25* derepression upon shift to methionine-free medium did not require CP1 (wild type versus *cep1*); however, in the *gcn4* background, expression was strongly CP1 dependent (*gcn4* versus *cep1 gcn4*). Thus, CP1 was not required for methionine-specific activation of *MET25* when GCN4 was present. That GCN4 becomes activated under these conditions was shown by the pattern of *HIS4* expression. *HIS4* RNA levels were inversely proportional to the growth phenotype of the strain; *HIS4* was not induced in the prototrophic wild-type strain, was partially induced in the *met16-47* and *met16-48* bradytrophs, and was fully induced in the auxotrophic *cep1* strain. Constitutive activation of GCN4 in *met16-47* and *met16-48* strains probably explains the superinduction of

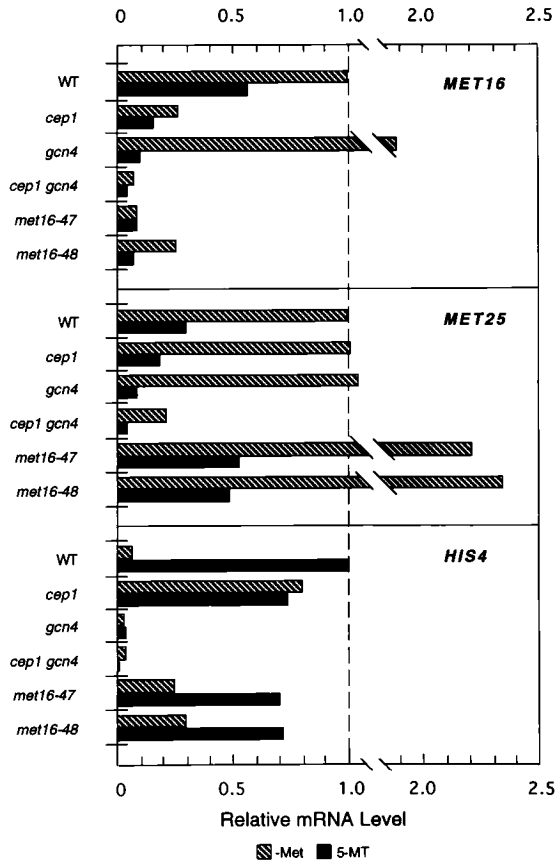

FIG. 5. General and pathway-specific control of *MET16* expression. Strains F113 (wild type), F212 (*gcn4*), K64-T1 (*cep1*), K63-T1 (*gcn4 cep1*), K55-M47 (*met16-47*), and K56-M48 (*met16-48*) were pregrown in $SCM_{1.0}$ and then shifted to SCM_0 or induced by 5-MT as for Fig. 2 and 4. RNA was isolated after 1 h (5-MT) or 2 h (− Met) and analyzed by Northern blotting. The graph shows the levels of *MET16*, *MET25*, and *HIS4* RNA in each strain under the indicated growth conditions. The data were first corrected for recovery of actin RNA and then normalized to the level of the corresponding transcript in the methionine-starved wild-type strain (*MET16* and *MET25*) or to that in the 5-MT-treated wild-type strain (*HIS4*).

MET25 upon methionine withdrawal (Fig. 3 and 5). Despite the involvement of general control in the modulating *MET16* and *MET25* expression, GCN4 is not essential for the methionine-specific (i.e., MET4-dependent) regulation of either gene, because the response of both genes to methionine withdrawal was intact in the *gcn4* mutant. Furthermore, activation of *MET16* or *MET25* by general control alone (i.e., by 5-MT) was not as effective as methionine limitation.

Disruption of *CEP1* leads to localized changes in *MET16* chromatin structure. MNase was used to probe the chromatin structure of the *MET16* locus. Chromatin in spheroplasts of cells grown under repressing or nonrepressing conditions was partially digested with MNase, and the cleavage sites were mapped by indirect end labeling (42). Under repressing conditions (+Met), wild-type and *cep1* chromatin contained essentially the same preferred MNase cleavage sites (Fig. 6). A regularly spaced pattern of cleavages extended from 950 bp upstream of the initiator ATG to 200 bp upstream of the CDEI site. Three of the sites (designated A, C, and D) were present in naked DNA, but their cleavage was enhanced in chromatin. In addition, two chromatin-specific sites, B and D′, were present in this region. In *cep1* cells, the regularly spaced cleavages continued into the *MET16* coding DNA. Sites E″ and F, sensitive in naked DNA, were protected, while chromatin-specific sites E and E′ appeared, flanking the CDEI. A significant difference in nuclease sensitivity was observed downstream of the CDEI in the region of DNA containing the putative TATA box (38). A cleavage site in naked DNA (site F) was accessible in wild-type chromatin but protected in *cep1* chromatin.

Noticeable changes in MNase sensitivity occurred when wild-type but not *cep1* cells were shifted to nonrepressing growth conditions. Site F became hypersensitive to digestion, and protection of site E″ was lost (Fig. 6, WT, −Met). In fact, the overall digestion pattern of derepressed wild-type chromatin in the CDEI region resembled that of naked DNA; i.e., sites D, E″, and F were present, and sites D′, E, and E′ were absent or reduced (Fig. 7). Downstream of site F, additional changes were observed. Cleavage at sites G′ and H′ was prominent, while sites G, H, and I were protected (Fig. 6). Since these downstream changes occurred in the transcribed region, they were probably a direct consequence of transcription.

Given the substrate specificity of MNase for nucleosome linker regions (42), we have interpreted differences in nuclease sensitivity to be caused by the presence or absence of nucleosomes (Fig. 6B). The 150- to 200-bp spacing of most cleavage sites is consistent with this interpretation. For the most part, the inferred positions of nucleosomes are independent of CP1; however, the localized changes in MNase sensitivity observed between strains and upon gene derepression could be explained by the altered association and/or loss of one or more nucleosomes flanking the CDEI site (see below).

DISCUSSION

All of our results are consistent with CP1 acting directly at *MET* gene promoters to facilitate initiation of transcription. Methionine-responsive, CP1-dependent expression of *MET16*, observed originally by Thomas et al. (40) and confirmed here, is conferred to a heterologous gene (*CYC1-lacZ*) by a 305-bp DNA fragment of the *MET16* 5′-flanking DNA. Expression of both the endogenous gene and the *MET16* UAS-driven reporter requires an intact CDEI site, and the effect of the most severe *cis*-acting CDEI mutation (*met16-48*) is equivalent to that of a *trans*-acting *cep1* mutation—both mutations abolish reporter gene activity and reduce native gene expression approximately 10-fold. In addition, the affinity of CP1 binding to mutated promoter elements positively correlates with the ability of those elements to activate expression of the *CYC1-lacZ* reporter. In particular, the *met16-39* mutation has an intermediate effect on both binding and expression. Lastly, the lack of both *MET16* RNA and *MET16* reporter gene expression in the *cep1* mutant is suppressed by PHO4, a factor known to act at the level of transcription initiation (33, 41). While *MET16* mRNA turnover rates were not directly measured (and measurement would be problematic given the low level of expression in *cep1* mutants), the combined data indicate that CP1 exerts its effect primarily, if not completely, at the level of mRNA synthesis.

The single exception to the correlation between CP1 binding affinity and reporter gene expression is *met16-33*. This mutation improves the similarity of the *MET16* element to the CDEI consensus sequence and increases CP1 binding affinity; however, reporter gene expression is reduced. Unlike the other mutations, the *met16-33* mutation alters nucleotides outside of the CDEI octamer. The decreased expression of the *met16-33*-driven reporter confirms that CP1 binding alone is insufficient for UAS activity and suggests that another *cis*-regulatory element abuts or overlaps the *MET16* CDEI site. Inspection of

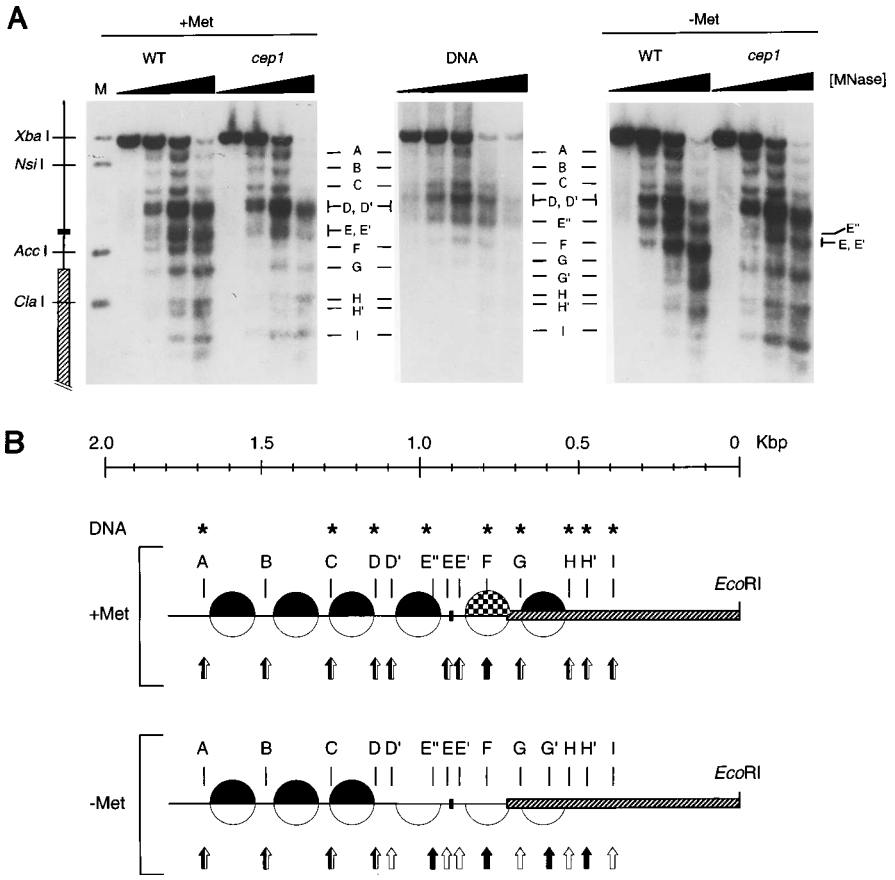

FIG. 6. Indirect end-label analysis of *MET16* chromatin structure in wild-type and *cep1* cells. Chromatin in strains K23-9A (wild type) and K23-9B (*cep1*) grown under repressed (+Met) or methionine-starvation (−Met) conditions was analyzed by the indirect end-labeling method described in Materials and Methods. The hybridization probe was complementary to sequences upstream of the *Eco*RI site. (A) Radiographic image of a typical blot, with MNase cleavage sites identified by capital letters. Lanes marked DNA contained deproteinized DNA treated with (from left to right) 1.5, 2.9, 5.9, 5.9, and 8.8 U of MNase per ml (the first three and last two digests, respectively, being performed on different days with different DNA preparations). Other lanes contained chromatin treated with (from left to right) 0, 9.2, 37, and 147 U of MNase per ml. Lane M contains a mixture of genomic DNA triple digests (*Eco*RI, *Xba*I, and either *Cla*I, *Acc*I, or *Nsi*I). (B) MNase cleavage map and inferred nucleosome positions. The *MET16* coding region is depicted by the diagonally striped bar, and the CDEI site is depicted by the small solid rectangle. Asterisks denote MNase cleavage sites in naked DNA. The upward-pointing arrows below each map indicate MNase cleavage sites in chromatin. Solid arrows denote cleavages detected primarily in wild-type chromatin, open arrows denote cleavages primarily in *cep1* chromatin, and half-open arrows denote cleavages observed for both strains. The semicircles represent nucleosomes and are drawn to scale (146 bp). Solid semicircles represent nucleosomes inferred to be present in the wild-type strain, and open semicircles represent nucleosomes inferred to be present in the *cep1* strain. The checkered semicircle represents a nucleosome in wild-type chromatin that only partially protects the underlying sequence (or is present only in a subpopulation of DNA molecules).

this region reveals homology to the 18-bp minimal UAS element identified in the *MET25* promoter; the sequence AAA TGR abuts the CDEI site in both cases. Significantly, this motif is found in four other *MET* genes, always in conjunction with a consensus CDEI but sometimes upstream and sometimes downstream (Fig. 8). The *met16-33* mutation alters three of the four most highly conserved nucleotides. Although corroborating evidence is lacking, it is tempting to speculate that the RA AATTAYR element is the binding site for *MET4*.

When *MET16* was cloned, Thomas et al. (38) noted the occurrence of a consensus GCN4-binding site in the promoter region but observed no gene activation upon arginine-limited growth. The present results demonstrate that *MET16* is activated in the general control response to tryptophan starvation. 5-MT treatment activates *MET16* in parallel with *HIS4*, and the transcription of both genes is GCN4 dependent. Maximal response of *MET16* but not *HIS4* to 5-MT also requires CP1. The activation of *MET16* by general control is distinguished from the methionine-specific activation pathway because it does not require *MET4*. Likewise, GCN4 is not required for the *MET4*-dependent response. Activation of the general control pathway may explain the slight discrepancy observed between the expression of the *MET16-CYC1-lacZ* reporter gene, which was totally CP1 dependent, and the endogenous *MET16* gene, which always produced low but reproducible levels of *MET16* RNA. For measuring reporter activity, *cep1* cells were grown in the presence of a nonrepressing concentration of methionine (50 μM), while for measuring endogenous *MET16* RNA levels, a starvation protocol was used. Conceivably, a full GCN4 response occurs only as intracellular methionine is totally depleted, a condition not encountered during growth in the presence of 50 μM methionine. If so, it may explain why Thomas et al. (40) found *MET16* expression to be totally dependent on *MET4*. In those experiments, cells were grown in the presence of 0.2 mM homocysteine and the general control pathway may not have been active.

Differential extents of GCN4 activation can also explain disagreement in the literature regarding *MET* gene expression in *cep1* (*cpf1*) mutants. In our experiments and in those of Mellor et al. (26), *MET25* expression was not greatly affected

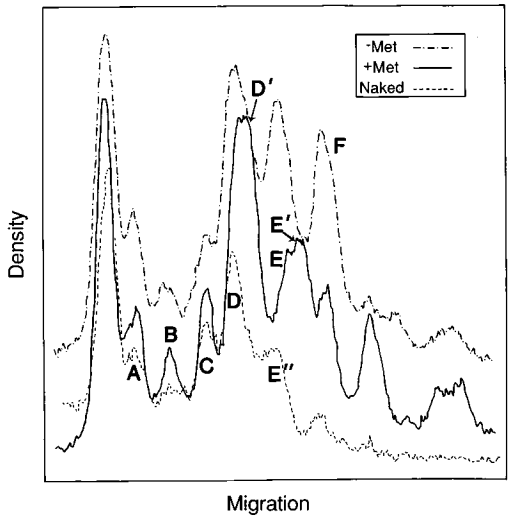

FIG. 7. Densitometer tracings of MNase cleavage maps illustrating differences in the region of the CDEI site. The autoradiograph shown in Fig. 6 was scanned, and tracings of lane 4 (WT, +Met, 37 U of MNase per ml), lane 12 (naked DNA, 5.9 U of MNase per ml), and lane 17 (WT, −Met, 37 U of MNase per ml) were superimposed. Note the accessibility of site F under both conditions and the gain of site E″ and loss of sites E and E′ upon gene derepression (−Met).

by the *cep1* mutation, while Thomas et al. (40) found *MET25* RNA levels to be reduced in a *cep1* mutant. Significantly, most of the *MET25* expression observed in the *cep1* strain under the conditions of our experiments (and presumably those of Mellor et al.) requires GCN4. This response, apparently not triggered under the growth conditions used by Thomas et al., obscures the requirement for CP1 in the pathway-specific response to methionine starvation. We would predict that other apparently CP1-independent *MET* genes (e.g., *MET2*, *MET3*, *SAM*1, and *SAM2*) require functional GCN4 for full expression in the absence of CP1. *MET16* requires CP1 for full induction (derepression) by either general control or the MET4 pathway, although the CP1 requirement is less stringent in the case of GCN4 activation. The failure of Kent et al. (21) to observe CP1 dependence in *MET16* expression may be due to experimental conditions which hyperactivate GCN4, thereby lessening dependence on CP1. Also, the extent of *MET16* induction upon methionine limitation was observed to be only twofold in that study (21), suggesting that full derepression was not obtained.

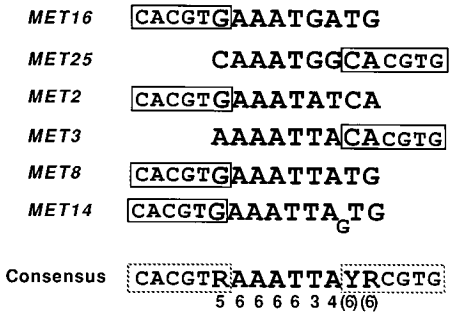

FIG. 8. CDEI-adjacent homology. Sequences flanking the upstream CDEIs of six *MET* genes were aligned and used to derive a consensus sequence for the putative *cis*-acting regulatory element. This element is found precisely in one of two positions relative to the CDEI site, whose palindromic core is boxed. The number of matches to the consensus sequence at each position is shown. A 1-bp insertion was allowed in the *MET14* sequence to maximize homology.

Our data do not directly address whether GCN4 activation of *MET16* and *MET25* is direct or indirect. Activation of *MET16* is likely to be direct, since the *MET16* upstream region contains a consensus GCN4-binding site and induction by 5-MT is independent of MET4. Analysis of *MET16* genes carrying site-directed mutations in the GCN4 site is needed to confirm the direct activation mechanism. The *MET25* promoter region does not contain a consensus GCN4-binding site (TGACTC), but the sequence TGACTA is present at position −257 relative to the translational ATG and also in opposite orientation at position −239. Mutagenesis experiments (17) would suggest that TGACTA would confer only weak GCN4 activation, and, indeed, 5-MT treatment elicits only a feeble *MET25* response. Alternatively, GCN4 could regulate *MET25* indirectly via MET4. Mountain et al. have found that the *MET4* promoter contains multiple GCN4 sites and is upregulated by general control (28). Constitutive activation of GCN4 (in a *gcd1* mutant) leads to derepression of *MET3* and *MET14* even though neither gene promoter contains GCN4-binding sites (29).

By traditional criteria, CP1 appears to lack an autonomous transcription activation domain. It has been suggested that CP1 somehow modulates chromatin structure to create an "active" configuration (25, 26). Consistent with this idea, strains that lack CP1 exhibit changes in the chromatin structure of CDEI-containing genes (21, 25, 26). The CP1-dependent changes that we detect in the chromatin structure of the *MET16* locus are limited to the immediate region of the CDEI site; the overall nuclease cleavage pattern of the *MET16* upstream region is essentially the same in the wild-type and *cep1* strains. Thus, CP1 does not act to set the phase of nucleosomes in this region, akin to the role played by REB1/GRF2 at the *GAL1-GAL10* intergenic region (13). The localized changes that are observed between *cep1* and wild-type strains are limited to the region immediately surrounding the CDEI site. The most significant difference is observed just downstream of the CDEI site, where an MNase-sensitive site in wild-type chromatin (site F) is strongly protected in the *cep1* mutant, consistent with the presence of a positioned nucleosome in the *cep1* strain and an altered ("loosened") association of this nucleosome with the underlying DNA in the wild-type strain. (Alternatively, two subpopulations of cells could exist, one containing and one lacking the nucleosome.) Since this region contains the putative TATA box and transcription initiation site (38), the presence of a stably positioned nucleosome (or other DNA-associated structure) would probably impede formation of the transcription initiation complex. Under nonrepressing conditions, the MNase-sensitive site F becomes hypersensitive to digestion but only in wild-type cells. The MNase digestion pattern of *cep1* chromatin is essentially unchanged upon shift to derepressing conditions. In addition, DNA just upstream of the CDEI in wild-type cells becomes more accessible to nuclease upon *MET16* derepression. The data can be accounted for by the loss of nucleosomes flanking the CDEI site. For the most part, our results are similar to data reported recently by Kent et al. (21). These authors also conclude that CP1 (CPF1) acts to maintain the region surrounding the *MET16* promoter CDEI site in a nucleosome-free state and that the same is true for *MET25* (21). Kent et al. did not analyze *MET16* chromatin structure upon gene derepression; however, as mentioned above, they did not find *MET16* transcription to be CP1 dependent.

The regulatory mechanisms governing expression of *MET16* are reminiscent of those which operate at the *HIS4* promoter. Devlin et al. (11) have shown that binding of the general regulatory factor RAP1 is required for both BAS1/BAS2-de-

pendent (basal control) and GCN4-dependent transcriptional activation of the *HIS4* gene. The RAP1-binding site partially overlaps a high-affinity GCN4-binding site and appears to increase the sensitivity of the surrounding region to digestion by MNase. Since binding sites for BAS1, BAS2, and GCN4 fall within this region, it has been suggested that RAP1 maintains the accessibility of these factors for their binding sites within chromatin. At the *MET16* promoter, CP1 is required for the normal operation of two activation mechanisms, appears to bind in close proximity to other factors, and increases the sensitivity of a nearby site in the surrounding DNA to MNase. CP1 is not responsible for setting the overall phasing of nucleosomes; rather, it appears to aid or catalyze the local reconfiguration of chromatin structure in the transcription initiation region. The molecular mechanism by which CP1 performs this function remains to be determined.

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REFERENCES

1. **Amasino, R. M.** 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. Anal. Biochem. **152:**304–307.
2. **Baker, R. E., M. Fitzgerald-Hayes, and T. C. 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.
3. **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.
4. **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 resistence. Mol. Gen. Genet. **197:**345–346.
5. **Bram, R. J., and R. D. Kornberg.** 1987. Isolation of a *Saccharomyces cerevisiae* centromere DNA-binding protein, its human homolog, and its possible role as a transcription factor. Mol. Cell. Biol. **7:**403–409.
6. **Buchman, A. R., W. J. Kimmerly, J. Rine, and R. D. Kornberg.** 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autononously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**210–225.
7. **Buchman, A. R., and R. D. Kornberg.** 1990. A yeast ARS binding protein activates transcription synergistically in combination with other weak activating factors. Mol. Cell. Biol. **10:**887–897.
8. **Cai, M., and R. W. Davis.** 1990. The yeast centromere binding protein CBF-I, a member of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell **61:**437–446.
9. **Chasman, D. I., N. F. Lue, A. R. Buchman, J. W. LaPointe, Y. Lorch, and R. D. Kornberg.** 1990. A yeast protein that influences the chomatin structure of UAS_G and functions as a powerful auxiliary gene activator. Genes Dev. **4:**503–514.
10. **Cherest, H., N. N. Thao, and Y. Surdin-Kerjan.** 1985. Transcriptional regulation of the *MET3* gene of *Saccharomyces cerevisiae*. Gene **34:**269–281.
11. **Devlin, C., K. Tice-Baldwin, D. Shore, and K. T. Arndt.** 1991. RAP1 is required for BAS1/BAS2- and GCN4-dependent transcription of the yeast *HIS4* gene. Mol. Cell. Biol. **11:**3642–3651.
12. **Donahue, T. F., P. J. Farabaugh, and G. R. Fink.** 1982. The nucleotide sequence of the *HIS4* region of yeast. Gene **18:**47–59.
13. **Fedor, M. J., N. F. Lue, and R. D. Kornberg.** 1988. Statistical positioning of nucleosomes by specific protein-binding to an upstream activating sequence in yeast. J. Mol. Biol. **204:**109–127.
14. **Fitzgerald-Hayes, M.** 1987. Yeast centromeres. Yeast **3:**187–200.
15. **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.
16. **Herrick, D., R. Parker, and A. Jacobson.** 1990. Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2269–2284.
17. **Hill, D. E., I. A. Hope, J. P. Macke, and K. Struhl.** 1986. Saturation mutagenesis of the yeast *his3* regulatory site: requirements for transcriptional induction and for binding by GCN4 activator protein. Science **234:**451–457.
18. **Hinnebusch, A. G.** 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52:**248–273.
19. **Hull, M. W., G. Thomas, J. M. Huibregtse, and D. R. Engelke.** 1991. Protein-DNA interactions *in vivo*—examining genes in *Saccharomyces cerevisiae* and *Drosophila melanogaster* by chromatin footprinting. Methods Cell Biol. **35:** 383–415.
20. **Ito, H., K. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
21. **Kent, N. A., J. S. H. Tsang, D. J. Crowther, and J. Mellor.** 1994. Chromatin structure modulation in *Saccharomyces cerevisiae* by centromere and promoter factor 1. Mol. Cell. Biol. **14:**5229–5241.
22. **Kerjan, P., H. Cherest, and Y. Surdin-Kerjan.** 1986. Nucleotide sequence of the *Saccharomyces cerevisiae MET25* gene. Nucleic Acids Res. **14:**7861–7871.
23. **Masison, D. C., and R. E. Baker.** 1992. Meiosis in *Saccharomyces cerevisiae* mutants lacking the centromere-binding protein CP1. Genetics **131:**43–53.
24. **Masison, D. C., K. F. O'Connell, and R. E. Baker.** 1993. Mutational analysis of the *Saccharomyces cerevisiae* general regulatory factor CP1. Nucleic Acids Res. **21:**4133–4141.
25. **McKenzie, E. A., N. A. Kent, S. J. Dowell, F. Moreno, L. E. Bird, and J. Mellor.** 1993. The centromere and promoter factor 1, CPF1, of *Saccharomyces cerevisiae* modulates gene activity through a family of factors including SPT2, RPD1, (SIN3), RPD3 and CCR4. Mol. Gen. Genet. **240:**374–386.
26. **Mellor, J., W. Jiang, M. Funk, J. Rathjen, C. A. Barnes, T. Hinz, J. H. Hegemann, and P. Philippsen.** 1990. CPF1, a yeast protein which functions in centromeres and promoters. EMBO J. **9:**4017–4026.
27. **Mellor, J., J. Rathjen, W. Jiang, and S. J. Dowell.** 1991. DNA binding of CPF1 is required for optimal centromere function but not for maintaining methionine prototrophy in yeast. Nucleic Acids Res. **19:**2961–2969.
28. **Mountain, H. A., A. Byström, and C. Korch.** 1993. The general amino acid control regulates *MET4*, which encodes a methionine-specific transcriptional activator of *Saccharomyces cerevisiae*. Mol. Microbiol. **7:**215–228.
29. **Mountain, H. A., A. S. Byström, J. Tang Larsen, and C. Korch.** 1991. Four major transcriptional responses in the methionine/threonine biosynthetic pathway of *Saccharomyces cerevisiae*. Yeast **7:**781–803.
30. **Ng, R., and J. Abelson.** 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:**3912–3916.
31. **Niedenthal, R., R. Stoll, and J. H. Hegemann.** 1991. In vivo characterization of the *Saccharomyces cerevisiae* centromere DNA element I, a binding site for the helix-loop-helix protein CPFI. Mol. Cell. Biol. **11:**3545–3553.
32. **O'Connell, K. F., and R. E. Baker.** 1992. Possible cross-regulation of phosphate and sulfate metabolism in *Saccharomyces cerevisiae*. Genetics **132:**63–73.
33. **Ogawa, N., and Y. Oshima.** 1990. Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2224–2236.
34. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. A laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Rothstein, R.** 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:**281–301.
36. **Schiestl, R. H., and R. D. Gietz.** 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:**339–346.
37. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
38. **Thomas, D., R. Barbey, and Y. Surdin-Kerjan.** 1990. Gene-enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae*. J. Biol. Chem. **265:**15518–15524.
39. **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.
40. **Thomas, D., I. Jacquemin, and Y. Surdin-Kerjan.** 1992. MET4, a leucine zipper protein, and centromere-binding factor 1 are both required for transcriptional activation of sulfur metabolism in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**1719–1727.
41. **Toh-e, A.** 1989. Phosphorus regulation in yeast, p. 41–52. *In* P. J. Barr, A. J. Brake, and P. Valenzuela (ed.), Yeast genetic engineering. Butterworth Publishers, Stoneham, Mass.
42. **Wu, C.** 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. Nature (London) **286:**854–860.