Multiple transcriptional activation complexes tether the yeast activator Met4 to DNA

Pierre-Louis Blaiseau¹ and
Dominique Thomas²

Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France

¹Present address: Institut Jacques Monod, 2 place Jussieu, 75251 Paris Cedex, France

²Corresponding author
e-mail: thomas@cgm.cnrs-gif.fr

The transcriptional regulation of the sulfur amino acid pathway in *Saccharomyces cerevisiae* depends on a single activator, Met4p, whose function requires different combinations of the auxiliary factors Cbf1p, Met28p, Met31p and Met32p. The first description of how these factors cooperate to activate transcription was provided by the identification of the Cbf1–Met4–Met28 complex which is assembled on the regulatory region of the *MET16* gene. In this paper, we demonstrate that other pathways are used to recruit Met4p on the 5' upstream region of the two genes, *MET3* and *MET28*. In these cases, Met4p is tethered to DNA through two alternative complexes associating Met4p with Met28p and either Met31p or Met32p. These complexes are formed over the AACTGTG sequence, a *cis*-acting element found upstream of several *MET* genes. The identification of a domain within Met4p that mediates its interaction with Met31p and Met32p allowed *in vivo* analysis of the specificity of the Met4p-containing complexes. The results therefore demonstrate that the co-regulation of a single gene network may be gained through different molecular mechanisms. In addition the sulfur system exacerbates the structural variety of the nucleoprotein complexes in which a single bZIP factor can be engaged.

Keywords: bZIP factors/heteromeric complex/sulfur amino acid metabolism/transcriptional activation/zinc finger factors

Introduction

Among the cellular responses to environmental, developmental or physiological modifications, the rapid changes of gene expression patterns are widely encountered. Many studies have pointed to the existence of a combinatorial control of gene expression. In most cases, the transcription of eukaryotic genes is regulated by multiple transcriptional activators or repressors, either because of the presence of multiple targets within the promoter regions or because several proteins assemble together to form oligomeric structures that are capable of recognizing specific *cis*-acting sequences (Tjian and Maniatis, 1994). A key issue for understanding the regulation of gene expression is to

decipher not only the mechanisms allowing the fine regulation of one particular gene but also those leading to the precise co-expression of a set of coordinately regulated genes in response to a single cellular signal.

For instance, in the yeast *Saccharomyces cerevisiae*, the study of which has led to much of our knowledge of the molecular mechanisms in transcription regulation, it is widely accepted that the mechanisms acting during the regulation of a gene network, such as a metabolic biosynthetic pathway, are identical for all of the regulated genes (Struhl, 1995). Therefore, studies performed on one particular gene are thought to recapitulate the overall regulatory mechanism. In yeast the metabolic pathway allowing the synthesis of the sulfur amino acids constitutes one of the few gene networks for which the transcriptional regulation has been systematically studied for almost all of the genes comprising the network (Thomas *et al.*, 1990; Kuras and Thomas, 1995b). This limited set of co-regulated genes has been shown to be sensitive mainly to one specific cellular signal, the increase in the intracellular concentration of *S*-adenosylmethionine (AdoMet), a widely used metabolite which can be formally considered as the end-product of the pathway (Thomas *et al.*, 1988). Quite surprisingly, both genetic and biochemical studies have revealed that the molecular mechanisms underlying this regulation differ among the co-regulated genes. Indeed, both the *cis*-acting sequences and the required *trans*-acting factors were shown to vary from one regulated gene to another (Kuras and Thomas, 1995b; Kuras *et al.*, 1996). However, it must be noted that only one protein endowed with intrinsic transcription activation function, the Met4p transcriptional activator has been identified so far, and that the expression of most, if not all, *MET* genes, is strictly dependent on the integrity of the Met4p factor (Thomas *et al.*, 1992).

Moreover, previous studies have outlined that in some cases, the transcriptional activation of the sulfur network is not achieved through the binding of isolated activators but rather results from the assembly of a hetero-oligomeric activation complex. In particular, use of both cellular extracts and purified recombinant proteins has shown that the transcriptional activation of the *MET16* gene arises through the assembly of a complex comprising three different subunits, the basic loop–helix–loop (bHLH)-containing factor Cbf1p and two basic leucine zipper (bZIP)-containing factors, Met4p and Met28p (Kuras *et al.*, 1996, 1997). The Cbf1–Met4–Met28 complex was shown to be assembled over the 5'-TCACGTG-3' motif, the unique *cis*-activating sequence present upstream of the *MET16* gene (O'Connell *et al.*, 1995). From these studies however, it was also hypothesized that the Cbf1–Met4–Met28 complex could only represent a particular class of heteromeric complexes formed on the promoter region of the structural *MET* genes.

Fig. 1. Transcription of *MET28* is dependent on Met31p and Met32p. Derepression kinetics of *MET28* transcription were monitored in wild-type cells and in cells lacking both Met31p and Met32p. The cells used were the strain CD164 (*met31::TRP1*, *met32::HIS3*) and its parental strain W303-1A. Cells were grown in 100 ml of B medium in the presence of a repressing amount (1 mM) of L-methionine as a sulfur source. When the cells reached a density of $\sim 10^7$ cells per ml, they were harvested by filtration and washed with 100 ml of B medium. The cells were then suspended in 100 ml of B medium without methionine and shaken at 28°C. Total RNA was then extracted at the indicated times after the shift, transferred onto a nylon membrane and hybridized to a *MET28* specific probe. The actin probe was used as a control of the amounts of RNA loaded.

This paper deals with the molecular mechanisms responsible for the transcriptional activation of the *MET28* gene. The experiments reported show that the recruitment of Met4p on the promoter region of this gene is achieved due to two other components, the recently identified Met31p and Met32p proteins. We demonstrate that, on such a promoter region, high-molecular-weight complexes are assembled which associate Met4p and Met28p itself to either Met31p or Met32p. Some of the structural features of these complexes are described and their involvement during the AdoMet-mediated regulation of the sulfur amino acid pathway are analyzed and discussed.

Results

Transcription activation of the *MET28* gene requires Met31p and Met32p

As reported previously, transcription of the *MET28* gene is negatively controlled by the increase of intracellular AdoMet and its activation is dependent on the integrity of Cbf1p, Met4p and Met28p itself (Kuras *et al.*, 1997). However, unlike observations with the promoter region of the *MET16* gene, when purified recombinant Cbf1p, Met4p and Met28p proteins were added together, they failed to assemble on the 5' upstream region of the *MET28* gene. This result therefore suggested that additional *trans*-acting factors are required for the expression of this gene. Two recently identified zinc-finger-containing proteins, Met31p and Met32p, were obvious candidates for such factors (Blaiseau *et al.*, 1997). Both Met31p and Met32p bind to the 5'-AAACTGTG-3' core sequence which is, besides the 5'-TCACGTG-3' element, the second regulatory element known to be involved in the regulation of several *MET* genes (Thomas *et al.*, 1989). A visual inspection revealed the presence of a AAACTGTG motif at position -145 (numbered relative to the ATG initiation codon) upstream of the *MET28* gene. It must be noted that, in this case, the motif is found in the opposite orientation. Transcription of the *MET28* gene was thus measured by Northern blot experiments in cells that did not express both Met31p and Met32p and compared with those measured in congenic wild-type cells. As shown in Figure 1, *MET28* transcripts are virtually undetectable in cells lacking both Met31p and Met32p. In contrast, the

Fig. 2. A high-molecular-weight complex is formed over the 5'-AAACTGTG-3' sequence found upstream of the *MET28* gene. Gel retardation assays were performed with a *MET28* radiolabeled probe corresponding to nucleotides -252 to -130. (A) Binding assays containing 40 μ g of wild-type cell extracts were incubated for 15 min at 4°C and then increasing amounts of a 24 bp oligonucleotide centered on the AAACTGTG motif were added. The reactions were incubated for another 15 min before being loaded on the gel. (B) The binding reactions were carried out with 40 μ g extracts from strains W303-1A (wild-type), CD158 (*met31::HIS3*), CD159 (*met32::HIS3*), CD179 (*met31::LEU2*, *met32::TRP1*), CD130-7D (*met28::LEU2*) and CD106 (*met4::TRP1*). 'CX' indicates the Met4 containing high-molecular-weight complexes.

single *met31* and *met32* disruption mutations affect the transcription of the *MET28* gene only slightly (data not shown). From this result, as well as from the previously reported studies (Kuras and Thomas, 1995b; Kuras *et al.*, 1996), it can be inferred that the transcriptional activation of the *MET28* gene requires at least five different *trans*-acting factors: Cbf1p, Met4p, Met28p, Met31p and Met32p.

Heteromeric complexes containing Met4p and Met28p associated with either Met31p or Met32p are formed on the *MET28* promoter region

To decipher the relations existing between the proteins described above and the *MET28* promoter region, mobility shift assays were first performed with various cellular extracts and a *MET28* probe corresponding to nucleotides -252 to -130. This probe contains the AAACTGTG sequence element but not the Cbf1p-binding site TCACGTG. Mobility shift assays performed with wild-type cell extracts show, in addition to several complexes of high electrophoretic mobility, two prominent complexes of low electrophoretic mobility (Figure 2A and B). Competition experiments performed using an excess of unlabeled oligonucleotides containing the AAACTGTG sequence demonstrated that the complex of higher molecular weight does form over the AAACTGTG motif (Figure 2A).

To identify the proteins responsible for the assembly of this complex, we then used a set of cellular extracts obtained from mutant strains, each bearing a disrupted allele of the *MET4*, *MET28*, *MET31* or *MET32* genes. An extract of the double *met31Δ*, *met32Δ* disrupted cells was

Fig. 3. Identification of the Met4-Met28-Met31 and Met4-Met28-Met32 complexes. Gel retardation assays were performed with a *MET28* radiolabeled probe corresponding to nucleotides -252 to -130. **(A)** The binding reactions were carried out with extracts from strains W303-1A (wild-type), CD106 (*met4::TRP1*), CD179 (*met31::LEU2, met32::TRP1*), CD158 (*met31::HIS3*) and from strains CD106, CD179 and CD158 but expressing a LexA-Met4, LexA-Met32 or Gad-Met31 fusion protein. All the reactions were carried out in the presence of 40 μ g of proteins except those performed with the CD158 cells which contained 70 μ g of proteins. When indicated, 1 μ l of a polyclonal antibody raised against LexA used at a 1:10 dilution was added. **(B)** A gel shift assay was carried out with extracts from cells W303-1A, CD130-7D (*met28::LEU2*) expressing a LexA-Met28 fusion protein and CD106 (*met4::TRP1*) expressing a LexA-Met4 fusion protein. An autoradiograph of the gel is displayed on the left. Proteins from the gel were transferred to a nitrocellulose based membrane and the LexA derivatives were detected with antibodies against LexA using ECL detection (WB, Western blot). 'CX' indicates the Met4-containing high-molecular-weight complexes.

also used. As shown in Figure 2B, the complex of lower electrophoretic mobility is not formed with extracts of the *met4Δ*, the *met28Δ* or the double *met31Δ, met32Δ* cells. In addition, intermediary patterns are observed with extracts of the cells bearing a single *met31Δ* or *met32Δ* mutation; in both cases, the complex of lower mobility is seen but its amount is 2- to 3-fold lower, compared with a wild-type extract. This suggests that Met31p and Met32p are equally capable of promoting the assembly of the complex of lower electrophoretic mobility. From all the results, it seemed possible that each of the proteins Met4p, Met28p, Met31p and/or Met32p could be found associated into a multi-subunit structure formed over the AAAGTGTG sequence which is present upstream of the *MET28* gene. To address this hypothesis directly, the *met4Δ* and the *met28Δ* cells were transformed using a plasmid expressing a LexA-Met4 fusion protein and a LexA-Met28 fusion protein, respectively. Mobility shift assays were performed with extracts of the transformed cells. In *met4Δ* cells, the expression of a LexA-Met4 derivative restores the formation of a complex of low electrophoretic mobility which, moreover, is supershifted by the addition of LexA antibodies to the binding reaction (Figure 3A), thus proving that Met4p is indeed a component of the complex. When the same experiment was performed with extracts of the *met28Δ* cells expressing a LexA-Met28 fusion protein, a complex of low mobility is seen but the addition of LexA antibodies to the binding reaction did not affect the mobility of this complex (data not shown). To determine whether Met28p actually contributes to the assembly of this complex, a Western blot analysis was carried out on a mobility shift assay. The complexes formed with extracts of cells expressing the LexA-Met28 fusion protein were separated by electrophoresis and transferred to a nitrocellulose membrane. As

shown in Figure 3B, LexA-Met28 was detected by the use of LexA antibodies therefore proving that Met28p does bind to the AAAGTGTG sequence through its association with the high-molecular-weight complex. For Met31p and Met32p, we used the double *met31Δ, met32Δ* mutant cells which were transformed either by a plasmid expressing Met31p fused to the Gal4p activation domain or by a plasmid expressing a LexA-Met32 derivative. In both cases, expression of the fusion proteins relieved the methionine auxotrophy of the double *met31Δ, met32Δ* mutant cells. Mobility shift assays were performed using extracts of the transformed cells. As shown in Figure 3A, expression of a Gal4-Met31 fusion protein leads to the formation of a high-molecular-weight complex which further exhibits a lowered mobility as compared with that of the complex observed in wild-type extracts, thereby proving that it contains Met31p. In contrast, the expression of a LexA-Met32 fusion protein in *met31Δ, met32Δ* cells results in a complex of high molecular weight, the mobility of which does not differ significantly from that of the complex seen with wild-type extract. The presence of Met32p within the complex was shown by the addition of antibodies against LexA to the binding reaction resulting in a supershift of the complex of lower electrophoretic mobility.

In summary, these series of experiments demonstrate that both Met4p and Met28p are capable of binding to the AAAGTGTG motif found upstream of the *MET28* gene when associated with either Met31p or Met32p. To provide further evidence that Met31p and Met32p might play redundant functions during the assembly of the complexes formed over the AAAGTGTG sequence, we took advantage of the strongly altered mobility displayed by a complex containing the Gal4-Met31 fusion protein. Mobility shift assays were performed with an extract made

Fig. 4. Two-hybrid analysis of the interactions established between the components of the Met4-Met28-Met31 and Met4-Met28-Met32 complexes. Schematic representation of the Gal4-Met4 fusion proteins are shown with the exact ends of the segments removed from the Met4 part. Plasmids encoding these derivatives were introduced into a *met4::TRP1* strain together with the pSH18-34 [LexA_{op}-LacZ fusion, (Gyuris *et al.*, 1993)] reporter construct and plasmid expressing either the LexA-Met31 or the LexA-Met32 fusion protein. The cells were grown in non-repressive growth conditions (0.05 mM L-Met) and β-galactosidase activities were assayed as described previously (Kuras and Thomas, 1995a). The data are expressed in nmol of substrate transformed per minute and per milligram of protein and are the averages of assays performed with four independent transformants (SD <15%).

from single *met31Δ*-disrupted cells expressing a Gal4-Met31 fusion protein. As shown in Figure 3A, this experiment shows two complexes of low mobility, one corresponding to the complex seen with the wild-type extract and the second corresponding to that seen with the extract of the double *met31Δ*, *met32Δ* mutant cells expressing the Gal4-Met31 derivative: this result indicates that both Met4-Met28-Met31 and Met4-Met28-Met32 complexes may exist simultaneously within the cells.

Met4p interacts with both Met31p and Met32p

Taken together, the results described above and the previous analyses of the Cbf1-Met4-Met28 complex (Kuras *et al.*, 1996) suggest that different molecular mechanisms are used to tether the Met4p transcriptional activator to DNA, depending on the promoter region: one acting through the Cbf1p-Met28p couple and the second requiring either Met31p or Met32p together with Met28p. As already demonstrated in the case of the Cbf1-Met4-Met28 complex, the targeting of Met4p to the AACTGTG sequence would imply that direct protein-protein interactions should be established between Met4p and the other components of the complexes containing either Met31p or Met32p. To determine whether interactions other than those made between Met4p and Met28p are established, we used the two-hybrid method (Fields and Song, 1989). The different fusion proteins were expressed in a *met4::TRP1* strain together with a LexA_{op}-LacZ reporter gene on a multi-copy plasmid. As shown in Figure 4, the activity of the LexA-Met31 and LexA-Met32 fusion proteins are stimulated 20- and 100-fold respectively by either the Gad-Met4-1 or the Gad-Met4Δ12 fusion proteins, the latter comprising a Met4p protein deleted from its activation domain. This suggests, therefore, that

Met4p indeed interacts with both Met31p and Met32p. As reported previously, Met4p also interacts with Met28p (Kuras *et al.*, 1996) but no interaction between Met28p and either Met31p or Met32p, or between Met31p and Met32p, could be detected using the two-hybrid method (data not shown). Next, we attempted to identify the regions of Met4p which are responsible for its interaction with Met31p and Met32p. The functional organization of Met4p has been studied in detail previously (Kuras and Thomas, 1995a), leading to the identification and delimitation of one activation domain and two regulatory regions (called the inhibitory region and the auxiliary domain) in addition to the bZIP motif which mediates the interaction of Met4p with both Cbf1p and Met28p. We focused our analysis on a specific region within Met4p spanning residues 375-430. Indeed, the expression of the Lex-Met4Δ38 derivative that does not contain this region was shown to be unable to complement the *met4Δ* mutation although the removal of these amino acids affected neither the transcription activation function of Met4p nor the AdoMet-mediated regulation of its activity (Kuras and Thomas, 1995a). We therefore constructed several Gal4-Met4 derivatives lacking either this particular region or various surrounding segments (all the further Gal4-Met4 derivatives derived from the Gad-Met4Δ12 protein that do not comprise the Met4p activation domain). As shown in Figure 4, all the Gal4-Met4 derivatives that do not contain the residues between the 375 and 403 amino acids fail to interact with both Met31p and Met32p, whereas they display interaction with Met28p. It is noteworthy that deletion of the Met4p bZIP domain, which abolishes the Met4p-Met28p interaction, decreases to a significant extent the interaction measured between Met4p and Met31p or Met32p. However, when the two-hybrid ana-

Fig. 5. Assembly of the Met4–Met28–Met31 and Met4–Met28–Met32 complexes requires both the 374–403 segment and the bZIP domain of Met4p. Gel retardation assays were performed with a *MET28*-radiolabeled probe corresponding to nucleotides –252 to –130. The binding reactions were performed with 40 µg of extracts from strains W303-1A (wild-type) and CD106 (*met4::TRP1*) expressing either the wild-type Met4p, the Met4Δ38 or the Met4Δ146 derivative, all from the *MET4* promoter on a centromeric plasmid. 'CX' indicates the Met4-containing high-molecular-weight complexes.

lysis was performed in *met4Δ*, *met28-1* mutant cells (strain CP86-10D), interactions between Met4p and both Met31p and Met32p were measured, thereby suggesting that Met28p might be required to stabilize the interaction established between Met4p and both Met31p and Met32p but is not essential for such protein–protein interactions.

The bZIP and the Met31-32-interacting domains of Met4 are both required for the assembly of the Met4–Met28–Met31(32) complexes

To examine the roles of the Met4p domains shown during the two-hybrid analysis, we determined the molecular species capable of binding to the *MET28* promoter region in cells expressing Met4p derivatives that do not comprise either the 375–403 region or the bZIP domain. *met4Δ* cells were transformed by autonomously replicating centromeric plasmids that expressed the different forms of Met4p from its own promoter, and extracts were made from the transformed cells. As shown in Figure 5, the expression of the wild-type Met4p from a plasmid leads to the formation of the complex of slower mobility, whereas this complex is not seen in cells expressing either the truncated form of Met4p lacking the bZIP domain or the Met4p-deleted derivative lacking the region spanning residues 375–403. The assays therefore confirm that two independent domains within Met4p are required for its tethering to the AAAGTGTG sequence by the Met28p, Met31p and Met32p proteins. It is moreover noteworthy that the bZIP domain of Met4p appears to be of crucial importance for its targeting to DNA, being necessary for the assembly of all the heteromeric complexes that have so far been demonstrated to allow the recruitment of Met4p to the promoter regions.

Met4 recruitment on the MET promoter regions

Results of the two-hybrid assays, as well as of the mobility shift experiments, suggested that the domain of Met4p

Fig. 6. Effects of Met4p mutations on *MET3* and *MET16* transcription activation. Derepression kinetics of *MET3* and *MET16* transcription were monitored in cells constitutively expressing Met28p (owing to the presence of *MET28* gene placed under the control of a *LEU2* promoter region) and a wild-type Met4p, or a modified Met4p lacking residues 374–430 or a modified Met4p lacking residues 616–666. The cells used were strain CC833-2B (*met4::TRP1*, *met28::LEU2*) transformed by plasmid pLeu2Met28, and by plasmid pM4-5, plasmid pM4Δ38 or plasmid pM4Δ146 (all the Met4 derivatives are expressed from the *MET4* promoter on centromeric plasmids). Total RNAs were extracted and analysed as described in Figure 1 with probes specific to the *MET3* and *MET16* genes.

contained within residues 375–403 could be specifically devoted to the assembly of the complexes associating Met4p with either Met31p or Met32p. Mutations that destroy this Met4p domain are therefore expected to prevent the transcriptional activation of the structural *MET* genes whose expression is dependent on the Met4–Met28–Met31(32) complexes, but not that of the *MET16* gene whose transcription is dependent on the assembly of the Cbf1–Met4–Met28 complex. To address this point, we compared the transcription of the *MET3* gene with that of the *MET16* gene in cells expressing a Met4p derivative that could no longer interact with Met31p and Met32p. The *MET3* gene was chosen because its 5' upstream region, like that of the *MET28* gene, contains the AAAGTGTG motif together with the TCACGTG sequence, the Cbf1p-binding site (Thomas and Surdin-Kerjan, 1997). Since all the Met4p-containing complexes also comprise Met28p, the *in vivo* analysis of the functional specificity of the 375–403 region of Met4p first required bypassing the regulatory cascade that places the expression of the *MET28* gene under the control of Met31p and Met32p.

The experiments were carried out using strain CC833-2B which carries two disruption mutations inactivating the chromosomal copies of both the *met4* and *met28* genes, but constitutively expresses Met28p owing to a *MET28* gene placed under the control of the *LEU2* promoter region. These cells were then transformed by centromeric plasmids expressing the wild-type Met4 protein, the Met4Δ38 protein (deleted from the 375–403 region) or a truncated form of Met4 (Met4Δ146) lacking the bZIP domain. Northern blot experiments were then performed with the transformed cells. As shown in Figure 6, in the presence of a wild-type form of Met4p, the constitutive expression of the *MET28* gene leads to similar derepression kinetics of the *MET3* and *MET16* genes. In contrast, the synthesis of a Met4p derivative lacking the Met31p–Met32p interacting region prevents the transcriptional activation of the *MET3* gene but not

Fig. 7. Met4p-containing complexes formed on the *MET3* promoter region. Gel retardation assays were performed with a *MET3*-radiolabeled probe corresponding to nucleotides –306 to –202. (A) Binding reactions performed with 40 µg extracts from strains W303-1A (wild-type), CD106 (*met4::TRP1*), CD130-7D (*met28::LEU2*) or CD179 (*met31::LEU2*, *met32::TRP1*). (B) Binding reactions performed with 40 µg of extract from strain CD106 (*met4::TRP1*) expressing a LexA–Met4 fusion protein in the presence or absence of 1 µl of a polyclonal anti-LexA antibody used at a 1/10 dilution. (C) Binding reactions performed with 40 µg of extracts from strain CC833-2B (*met4::TRP1*, *met28::LEU2*) expressing Met28p under the *LEU2* promoter from a centromeric plasmid and either the wild-type Met4p, the Met4Δ38 or the Met4Δ146 derivative, all from the *MET4* promoter on a centromeric plasmid. ‘CX’ indicates the Met4-containing high-molecular-weight complexes.

that of the *MET16* gene. Finally, neither the *MET3* nor the *MET16* gene are expressed in cells expressing a Met4p derivative lacking the bZIP domain as expected from the involvement of this domain in the assembly of both the Cbf1–Met4–Met28 and the Met4–Met28–Met31(32) complexes. In addition, this experiment revealed that the transcriptional activation of the *MET16* gene does not depend directly on the integrity of the Met31p and Met32p factors. Indeed, although *MET16* transcripts cannot be detected in double *met31Δ*, *met32Δ* mutant cells (data not shown), all the results reported above show that such an impairment was an indirect consequence of the lack of transcription of the *MET28* gene in the absence of Met31p and Met32p. Accordingly, the expression of the *MET28* gene under the control of the *LEU2* promoter region results in the transcription activation of the *MET16* gene in *met31Δ*, *met32Δ* mutant cells (data not shown).

To further confirm that *MET3* gene expression depends on the assembly of the Met4–Met28–Met31 and Met4–Met28–Met32 complexes on its 5′ upstream region, mobility shift assays were carried out with extracts of different strains and with a *MET3* probe containing the AAACGTG sequence (corresponding to nucleotides –306 to –202). As indicated by an arrow in Figure 7A, a complex of low electrophoretic mobility is visualized, which is formed only in wild-type cells and not in *met4Δ*, *met28Δ* or *met31Δ*, *met32Δ* cells. As for the *MET28* probe, overexpression of a LexA–Met4 derivative resulted in the formation of a complex which could be supershifted by the addition of anti-LexA antibodies (Figure 7B). In addition mobility shift assays performed with extracts of CC833-2B cells synthesizing Met28p showed constitutively that this complex was formed only when such cells expressed the wild-type form of Met4p and not when they expressed either the Met4Δ38 or the Met4Δ146 derivative (Figure 7C). All the results thus suggest that Met4p is

recruited to the *MET3* promoter region in a way similar to that allowing its targeting on the *MET28* promoter region.

In summary, mobility shift assays, as well as Northern blot analyses, prove that the Met4p region spanning residues 375–403 is required for the assembly of both the Met4–Met28–Met31 and the Met4–Met28–Met32 complex but not for assembly of the Cbf1–Met4–Met28 complex. In addition, these experiments also demonstrate that the Met31p, Met32p-mediated recruitment of Met4p to DNA is not restricted to the regulatory *MET28* gene.

Relationships between Cbf1p and the Met4–Met28–Met31(32) complexes

The 5′ upstream regions of both *MET3* and *MET28* genes comprise the AAACGTG motif together with the Cbf1p DNA-binding site, TCACGTG. However, contrary to what was observed for the *MET16* gene, *in vitro* reconstitution experiments failed to demonstrate the possibility of the Cbf1–Met4–Met28 complex assembling on the TCACGTG motif present upstream of the *MET3* and *MET28* genes (Kuras *et al.*, 1997; L.Kuras, unpublished). In addition, it must be remembered that Northern blot experiments have revealed that inactivation of the Cbf1p-encoding gene decreases the expression of *MET3* and *MET28* only weakly (Kuras and Thomas, 1995b; Kuras *et al.*, 1997). Regarding the identification of the Met4–Met28–Met31 and Met4–Met28–Met32 complexes reported here, the exact function of Cbf1p bound on the promoter regions of these two genes therefore remained to be deciphered. To gain further insight into this point, mobility shift assays were carried out using a *MET28* probe that contained both the AAACGTG and TCACGTG motifs (corresponding to nucleotides –299 to –134) and various cellular extracts. As shown in Figure 8A, three complexes of low mobility can be seen with wild-type extracts. When the assays were performed with cells expressing a LexA–Cbf1 protein

Fig. 8. (A) Gel retardation assays were performed with a *MET28*-radiolabeled probe corresponding to nucleotides –299 to –134 and containing both the TCACGTG and AAAGTGTG elements. The binding reactions were performed with 40 µg of extracts from strains W303-1A (wild-type), CD106 (*met4::TRP1*) expressing a LexA–Met4 fusion protein, CD130-7D (*met28::LEU2*), CD179 (*met31::LEU2, met32::TRP1*) or CC718-1A (*cbf1::TRP1*) expressing or not a LexA–Cbf1 fusion protein. When indicated 1 µl of a polyclonal anti-LexA antibody used at a 1:10 dilution was added. (B) Competition experiments performed with a *MET28*-radiolabeled probe corresponding to nucleotides –299 to –134. Binding assays containing 40 µg of extracts from strain CD106 expressing the wild-type Met4p from a centromeric plasmid were incubated for 15 min at 4°C and then increasing amounts of a 24 bp oligonucleotide centered on the AAAGTGTG motif were added. The reactions were incubated for another 15 min before being loaded on the gel. (C) The experiments were as in (B) except that the *MET28* probe corresponds to nucleotides –252 to –130 and does not contain the TCACGTG motif. ‘CX’ indicates the Met4-containing high-molecular-weight complexes.

fusion, all three complexes displayed a lowered mobility and, in addition, were supershifted by the addition of antibodies against LexA to the binding reactions, therefore proving that all these complexes comprised Cbf1p. In contrast, when the assays were performed with extracts of cells expressing the LexA–Met4 derivative, only the complex of lower mobility displayed a modified electrophoretic mobility and was, moreover, supershifted by the presence of anti-LexA antibodies. In addition, this complex was specifically not seen when the assays were carried out with either the *met28Δ*- or the *met31Δ, met32Δ*-disrupted cells (Figure 8A). This indicated that the complex of lower electrophoretic mobility corresponds to the binding of Cbf1p together with the Met4–Met28–Met31(32) complexes on the same DNA molecules. Since the binding reactions were performed in the presence of an excess of the labeled probe, the fact that the experiments did not show evidence of a complex that could be ascribed to the binding of the Met4–Met28–Met31(32) complexes without Cbf1p would suggest that the binding of Cbf1p to DNA could enhance the affinity of the Met4–Met28–Met31(32) complexes for its own target site. To provide more evidence of such a hypothesis, competition assays were performed using a *MET28* probe comprising only the AAAGTGTG sequence element and a *MET28* probe containing the two motifs TCACGTG and AAAGTGTG. The binding reactions were carried out using cells expressing the wild-type Met4p from a centromeric plasmid to enhance the amount of assembled complexes. The reactions were allowed to reach equilibrium and increasing amounts of oligonucleotides containing the AAAGTGTG sequence were added. As shown in Figure 8B and C, the Met4p-containing complexes are displaced for a lower amount of oligonucleotides in the absence of the Cbf1p-binding

site than in the presence of Cbf1p bound to the DNA. The binding of Cbf1p to its target thus appears to stabilize the interactions established between the Met4–Met28–Met31(32) complexes and their target site.

Discussion

The gene network responsible for the synthesis of sulfur amino acids in yeast comprises ~25 genes scattered throughout the genome. Kinetics analyses of the transcription rates have demonstrated that most of these genes are co-regulated precisely (Kuras and Thomas, 1995b). Previous studies have, however, indicated that such a coordinated regulation could be achieved through different molecular mechanisms depending on the target gene. Two *cis*-acting elements were indeed identified (the TCACGTG and AAAGTGTG motifs) but all *MET* genes do not contain these two sequences in their 5' upstream region. Likewise, the expression of the *MET* genes was shown to be not uniformly affected by mutations inactivating the *trans*-acting factors Cbf1p, Met28p, Met31p and Met32p (Kuras and Thomas, 1995b; Blaiseau *et al.*, 1997). Actually, the only factor that was demonstrated to be invariably required for expression of all the *MET* genes was the bZIP factor Met4p. Met4p is also the single *trans*-acting factor known to possess intrinsic transcription activation capacities. Indeed, one-hybrid assays revealed that neither Cbf1p nor the factors Met28p, Met31p and Met32p could function as transcriptional activators (Thomas *et al.*, 1992; Kuras *et al.*, 1996; Blaiseau *et al.*, 1997).

First insights into how all these proteins cooperate were provided by the identification of the heteromeric transcription activation complex comprising Cbf1p, Met4p and Met28p, which was shown to assemble on the

Fig. 9. (A) Schematic representation of the functional organization of Met4 deduced from Kuras and Thomas (1995a) and Thomas *et al.* (1995), and from the results reported above. (B) Modalities of Met4p recruitment to the DNA depending on the *MET* gene. Two alternative hypotheses about the Met31/Met32-containing complexes are depicted (see text).

Table I. Yeast strains

Strain	Genotype	Reference
CC718-1A	<i>MATa, ade2, his3, leu2, trp1, ura3, cbf1::TRP1</i>	Kuras <i>et al.</i> (1996)
CC833-2B	<i>MATα, ade2, his3, leu2, trp1, ura3, met4::TRP1 met28::LEU2</i>	Kuras <i>et al.</i> (1996)
CD106	<i>MATa, ade2, his3, leu2, trp1, ura3, met4::TRP1</i>	Thomas <i>et al.</i> (1992)
CD130-7D	<i>MATα, ade2, his3, leu2, trp1, ura3, met28::LEU2</i>	H.Chérest (unpublished)
CD158	<i>MATa, ade2, his3, leu2, trp1, ura3, met31::HIS3</i>	Blaiseau <i>et al.</i> (1997)
CD159	<i>MATa, ade2, his3, leu2, trp1, ura3, met32::HIS3</i>	Blaiseau <i>et al.</i> (1997)
CD164	<i>MATα, ade2, his3, leu2, trp1, ura3, met31::TRP1, met32::HIS3</i>	this study
CD179	<i>MATα, ade2, his3, leu2, trp1, ura3, met31::LEU2 met32::TRP1</i>	this study
CP86-10D	<i>MATa, ade2, his3, leu2, trp1, ura3, met4::TRP1, met28-1</i>	H.Chérest (unpublished)
W303-1A	<i>MATa, ade2, his3, leu2, trp1, ura3</i>	Bailis and Rothstein (1990)

TCACGTG motif present upstream of the *MET16* gene (Kuras *et al.*, 1997). Moreover, this set of experiments demonstrated that the high-affinity tethering of Met4p to the *MET16* promoter region is achieved through the stimulation, by Met28p, of the DNA-binding of Cbf1p (Kuras *et al.*, 1997). However, from these experiments, it was also clear that the overall regulation of the sulfur network could not be ascribed solely to the assembly of the Cbf1–Met4–Met28 complex since this complex did not form on the 5' upstream region of genes such as *MET28* and *MET3*. The studies reported here provide evidence that different combinations of *trans*-acting factors are used to tether Met4p to the DNA. Indeed, we show that Met4p is recruited to the promoter region of the *MET28* and *MET3* genes through its association with Met28p and either Met31p or Met32p in high-molecular-weight complexes. Met31p and Met32p are two small zinc finger proteins which were primarily identified as

factors capable of recognizing the AAACGTG motif present upstream of several *MET* genes. Although further experiments with purified recombinant proteins are required to provide a more accurate description of the Met4–Met28–Met31(32) complexes, especially regarding the stoichiometry of each of the components, all the reported results are consistent with the assembly of two different complexes, containing either Met31p or Met32p. This possibility is further sustained by the previously reported genetic analyses of the *met31Δ*- and *met32Δ*-mutant strains which revealed the functional redundancy of these two proteins (Blaiseau *et al.*, 1997).

Two-hybrid assays allowed determination of the binary protein–protein contacts required to assemble the Met4–Met28–Met31 and Met4–Met28–Met32 complexes. Evidence for direct protein–protein contact could not be found between Met28p and either Met31p or Met32p. In contrast, as in the case of the Cbf1–Met4–Met28 complex, Met4p

appears to play a pivotal role, interacting with both Met28p and either Met31p or Met32p. A functional dissection of Met4p further led to the identification of a particular domain within Met4p which specifically mediates its interaction with Met31p and Met32p. As illustrated in Figure 9A, this domain encompasses residues 375–403 of Met4p. The removal of these residues leads to a Met4 derivative which is no longer capable of interacting with Met31p and Met32p or being recruited to the AAAGTGTG motif. Moreover, the functional specificity of this domain was demonstrated *in vivo*. In cells constitutively expressing Met28p, the synthesis of a Met4 derivative lacking the 366–374 segment is capable of sustaining the transcriptional activation of the *MET16* gene (which depends on the Cbf1–Met4–Met28 complex) but not that of the *MET3* gene. Likewise, Northern blot analysis confirmed both the mobility shift and the two-hybrid assays by showing that the bZIP domain of Met4p is required for the transcriptional activation of both the *MET3* and *MET16* genes. The bZIP domain of Met4p is therefore required for the assembly of the Cbf1–Met4–Met28 complexes on the one hand and for the assembly of the Met4–Met28–Met31 and Met4–Met28–Met32 complexes on the other hand. It is thus tempting to postulate that in fact, it is the bZIP heterodimer Met4–Met28 which is recruited to two different DNA targets through its association with either a bHLH factor (Cbf1) or zinc finger factors (Met31p, Met32p), demonstrating once again the versatility of the protein–protein interactions that can be established between DNA-bound factors.

Since the AAAGTGTG motif is found associated with the TCACGTG sequence element in the 5' upstream region of both the *MET28* and *MET3* genes, we carried out preliminary experiments to decipher the relationships established between the Met4–Met28–Met31(32) complexes and Cbf1p on such regulatory regions. The results of mobility shift assays favor the existence of a direct interaction between Cbf1p and the complexes assembled over the AAAGTGTG sequence. However, the results should be interpreted with caution because of the resolution of the assays performed with the cellular extracts. In any case, competition assays suggest unequivocally that the binding of Cbf1p in the vicinity of the AAAGTGTG sequence enhances the DNA-binding affinity of the Met4–Met28–Met31(32) complexes. This could be accounted for by the formation of a large complex associating all the described factors, in this hypothesis Met4p being tethered to the DNA through two different anchors. Alternatively, the increase in the DNA-binding affinity of the Met4–Met28–Met31(32) complexes might be the result of a localized modification of the DNA structure induced by the binding of Cbf1p to its site. Cbf1p is indeed known to bend DNA upon binding (Niedenthal *et al.*, 1993) and such a function was taken into account to explain its role at the centromeres (Wilmen *et al.*, 1994; Meluh and Koshland, 1997). Figure 9B depicts the plausible modalities of Met4p tethering to the DNA.

The promoter regions of most eukaryotic genes have binding sites for more than one activator and detailed studies have provided evidence that transcriptional activators could synergize each other to enhance transcription activation. Transcriptional synergy could be ascribed to cooperative binding of the activators to their sites as well

as to interactions between proteins that bind nonadjacent sites and to protein-induced DNA binding (Giese *et al.*, 1995; Kim and Maniatis, 1997). Although some aspects of the transcription activation complexes described here are reminiscent of that observed for the so-called 'enhanceosomes', the sulfur network displays specific features. Certainly, the most striking feature is the number of factors that cooperate to allow the recruitment of only one transcriptional activator, Met4p. Therefore, the formation of the highly ordered nucleoprotein complexes involved in the sulfur network should be understood in terms different from synergy. Rather, the construction of such multi-subunit structures, associated with the regulatory loops that place some of the components of the complexes under the control of others (Kuras *et al.*, 1997; this study), could be used by the cells to install a highly precise and dynamic device regulating the sulfur flux.

In addition, it should be noted how the sulfur gene network exacerbates the structural diversity of the nucleoprotein complexes that can accommodate the DNA targeting of a single transcriptional activator. Indeed, the description of the Met4p-containing complexes shows that, in one case, this bZIP factor can associate with both a bZIP factor (Met28p) and a bHLH factor (Cbf1p), whereas in the other case, it is capable of interacting with both a bZIP factor (Met28p) and a zinc-finger-containing protein (Met31p or Met32p). To date we are unable to understand the physiological reasons for constructing such different transcription activation complexes to regulate a single metabolic pathway. However, it seems reasonable to postulate that the flexibility exhibited by Met4p in interacting with various DNA-binding factors, and thereby with different *cis*-acting regulatory elements, might help the cells to establish the regulatory cross-talks necessary for its overall metabolic homeostasis.

Materials and methods

Yeast strains and media

The *S.cerevisiae* strains used in this work are listed Table I. Standard yeast media were prepared as described by Chérest and Surdin-Kerjan (1992). *Sacharomyces cerevisiae* was transformed after acetate chloride treatment as described by Gietz *et al.* (1992). Genetic crosses, sporulation, dissection and scoring of nutritional markers were as described by Sherman *et al.* (1979).

Plasmids

Met4 derivatives. The modified Met4 proteins were expressed in yeast from pProM4-0, an autonomously replicating centromeric plasmid containing the *URA3* gene and 600 bp of the 5' upstream region of *MET4* followed by a multiple cloning site. pProM4-0 was cleaved by *EcoRI* and *BamHI* and ligated with *EcoRI* and *BamHI* fragments from pLexM4-3 (Thomas *et al.*, 1992) and pLexM4Δ38 (Kuras and Thomas, 1995a) yielding plasmids pM4Δ146 and pM4Δ38, respectively. The pM4-5 plasmid allowing the expression of the wild-type form of Met4 was as described in Kuras and Thomas (1995a).

Gal4–Met4 derivatives. All the plasmids pGADM4Δi that express derivatives of Met4p fused to the activation domain of Gal4p were derived from plasmid pGADM4Δ12 (Kuras and Thomas, 1995a) which corresponds to the fusion of Met4p deleted from its activation domain (residues 79–180) to the Gal4 activation domain. Plasmids pGADM4Δ103, pGADM4Δ108, pGADM4Δ116, pGADM4Δ137 and pGADM4Δ138 were obtained by ligating the *NcoI*–*BamHI* fragments of pLexM4-3, pLexM4Δ8, pLexM4Δ16, pLexM4Δ37 and pLexM4Δ38, respectively (Kuras and Thomas, 1995a) into pGADM4Δ12 cleaved by *NcoI* and *BamHI*, thus resulting in plasmids expressing Gal4–Met4 fusion proteins deleted for both the activation domain and a second

region of Met4. The plasmids pGADM4Δ27 and pGADM4Δ28 were constructed, by ligating the *EcoRI*–*Bam*HI fragments of pLexM4Δ27 and pLexM4Δ28, respectively (Kuras and Thomas, 1995a) into pGADM4Δ12 that had been cleaved by *EcoRI* and *Bam*HI.

pLeu2Met28. This plasmid, allowing the constitutive expression of Met28p, derives from pProLeu2, an autonomously replicating centromeric plasmid containing the *HIS3* gene and 650 bp of the 5' upstream region of *LEU2* followed by a multiple cloning site. pProLeu2 was cleaved by *Sma*I and *Sal*I and ligated with the *Sma*I–*Sal*I fragment from pLex Met28-2 (Kuras and Thomas, 1996).

The plasmids allowing the expression of the LexA–Met28, LexA–Met31, LexA–Met32 and Gal4–Met31 have been described previously (Kuras *et al.*, 1996; Blaiseau *et al.*, 1997).

Northern blot analyses

Northern blotting was performed as described by Thomas (1980), using total cellular RNA extracted from yeast as described by Hoffman and Winston (1987) and oligolabeled probes (Hodgson and Fisk, 1987).

Electrophoretic mobility shift assays

The cellular extracts were prepared as described previously (Kuras *et al.*, 1996) except that a phosphate-based buffer was used: 100 mM Na₂HPO₄ pH 8.0, 1 mM EDTA, 25 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 12 mM β-mercaptoethanol and 10% glycerol.

For the mobility shift assays, the binding reactions mixtures (20 μl volume) contained 25 mM HEPES pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, 5 mM MgCl₂ and 3 μg of poly(dI–dC)–poly(dI–dC). The amount of protein used in each binding is indicated in the figure legends. Probes were labeled by filling in the ends with the Klenow fragment and [α-³²P]dATP (800 Ci/mmol; Amersham). Approximately 20 000 c.p.m. of probe (~0.5 ng) was used in each binding mixture. Samples were incubated for 30 min on ice before being loaded onto a 5% polyacrylamide gel in 0.25× TBE (22 mM Tris pH 8.3, 22 mM boric acid, 0.6 mM EDTA) and electrophoresed at 15 V/cm at 7°C. Gels were run for 4 h, dried and autoradiographed for 15 h with an intensifying screen.

β-galactosidase assays

β-galactosidase activity was assayed as described previously (Kuras and Thomas, 1995a). Protein concentrations were estimated by the method described by Lowry *et al.* (1951).

Acknowledgements

We are indebted to Yolande Surdin-Kerjan for her constant support and encouragement. We are grateful to Pascal Maire for helpful discussions. We thank Laurent Kuras, Astrid Rouillon and Caroline Peyraud for providing us with plasmids. We thank Dr P.L. Moreau for providing us with the LexA antibodies. This work was supported by the Centre National de la Recherche Scientifique and the Association de la Recherche sur le Cancer.

References

Bailis, A.M. and Rothstein, R. (1990) A defect in mismatch repair in *Saccharomyces cerevisiae* stimulates ectopic recombination between homeologous genes by an excision repair dependent process. *Genetics*, **126**, 535–547.

Blaiseau, P.L., Isnard, A.D., Surdin-Kerjan, Y. and Thomas, D. (1997) Met31p and Met32p, two related zinc finger proteins are involved in transcriptional regulation of the yeast sulfur amino-acid metabolism. *Mol. Cell. Biol.*, **17**, 3640–3648.

Chérest, H. and Surdin-Kerjan, Y. (1992) Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. *Genetics*, **130**, 51–58.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Giese, K., Kingsley, C., Kirshner, J.R. and Grosschedl, R. (1995) Assembly and function of a TCRα enhancer complex is dependent on LEF-1 induced DNA bending and multiple protein–protein interactions. *Genes Dev.*, **9**, 995–1008.

Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Improved

method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425–1426.

Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993) Cdi1, a human G₁ and S phase protein phosphatase that associates with Cdk2. *Cell*, **74**, 205–214.

Hodgson, C.P. and Fisk, R.Z. (1987) Hybridization probe size control: optimized 'oligolabelling'. *Nucleic Acids Res.*, **15**, 6295.

Hoffman, C.S. and Winston, F. (1987) A ten minute DNA preparation from yeast releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, **57**, 267–272.

Kim, T.K. and Maniatis, T. (1997) The mechanism of transcriptional synergy of an *in vitro* assembled interferon β enhanceosome. *Mol. Cell*, **1**, 119–129.

Kuras, L. and Thomas, D. (1995a) Functional analysis of Met4, a yeast transcriptional activator responsive to S-adenosylmethionine. *Mol. Cell. Biol.*, **15**, 208–216.

Kuras, L. and Thomas, D. (1995b) Identification of the yeast methionine biosynthetic genes that require the centromere factor 1 for their transcriptional activation. *FEBS Lett.*, **367**, 15–18.

Kuras, L., Chérest, H., Surdin-Kerjan, Y. and Thomas, D. (1996) A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. *EMBO J.*, **15**, 2519–2529.

Kuras, L., Barbey, R. and Thomas, D. (1997) Assembly of a bZIP/bHLH transcription activation complex: formation of the yeast Cbf1/Met4/Met28 complex is regulated through Met28 stimulation of Cbf1 DNA binding. *EMBO J.*, **16**, 2441–2451.

Lowry, O.H., Rosebrough, J.H., Farr, A.L. and Randall, J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Meluh, P.B. and Koshland, D. (1997) Budding yeast centromere composition and assembly as revealed by *in vivo* cross-linking. *Genes Dev.*, **11**, 3401–3412.

Niedenthal, R.K., Sen-Gupta, M., Wilmen, A. and Hegemann, J.H. (1993) CPFI protein induced bending of yeast centromere DNA element 1. *Nucleic Acids Res.*, **21**, 4726–4733.

O'Connell, K., Surdin-Kerjan, Y. and Baker, R.E. (1995) Role of the *Saccharomyces cerevisiae* general regulatory factor CP1 in methionine biosynthetic gene transcription. *Mol. Cell. Biol.*, **15**, 1879–1888.

Sherman, F., Fink, G.R. and Hicks, J.B. (1979) *Methods in Yeast Genetics: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Struhl, K. (1995) Yeast transcriptional regulatory mechanisms. *Annu. Rev. Genet.*, **29**, 651–674.

Thomas, D. and Surdin-Kerjan, Y. (1997) Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.*, **61**, 503–532.

Thomas, D., Rothstein, R., Rosenberg, N. and Surdin-Kerjan, Y. (1988) SAM2 encodes the second methionine S-adenosyl transferase in *Saccharomyces cerevisiae*: physiology and regulation of both enzymes. *Mol. Cell. Biol.*, **8**, 5132–5139.

Thomas, D., Chérest, H. and Surdin-Kerjan, Y. (1989) Elements involved in S-adenosylmethionine mediated regulation of the *Saccharomyces cerevisiae* MET25 gene. *Mol. Cell. Biol.*, **9**, 3292–3298.

Thomas, D., Barbey, R. and Surdin-Kerjan, Y. (1990) Gene–enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae*. Study of the 3'-phosphoadenylylsulfate reductase structural gene. *J. Biol. Chem.*, **265**, 15518–15524.

Thomas, D., Jacquemin, I. and Surdin-Kerjan, Y. (1992) MET4, a leucine zipper protein and centromere binding factor I, are both required for transcriptional activation of sulfur metabolism in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **12**, 1719–1727.

Thomas, D., Kuras, L., Barbey, R., Chérest, H., Blaiseau, P.L. and Surdin-Kerjan, Y. (1995) Met30, a yeast transcriptional inhibitor that responds to S-adenosylmethionine, is an essential protein with WD40 repeats. *Mol. Cell. Biol.*, **15**, 6526–6534.

Thomas, P.S. (1980) Hybridization of denatured DNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl Acad. Sci. USA*, **77**, 5201–5205.

Tjian, R. and Maniatis, T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.

Wilmen, A., Pick, H., Niedenthal, R.K., Sen-Gupta, M. and Hegemann, J.H. (1994) The yeast centromere CDE1/Cpf1 complex: differences between *in vitro* binding and *in vivo* function. *Nucleic Acids Res.*, **22**, 2791–2800.

Received July 23, 1998; revised September 9, 1998;
accepted September 10, 1998