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

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Transcriptional activation of sulfur amino acid metabolism in yeast is dependent on a multi-functional factor, the centromere-binding factor 1 (Cbf1) and on two specific transcription factors, Met4 and Met28. Cbf1 belongs to the basic helix–loop–helix DNA-binding protein family while Met4 and Met28 are two basic leucine zipper (bZIP) factors. We have shown previously that in cell extracts, the three factors are found in a high molecular weight complex. By using mobility shift assays, we report here that the *in vitro* reconstitution of the Cbf1–Met4–Met28 complex on *MET16*_{UAS} can be obtained with purified recombinant proteins. DNase I protection experiments confirm that the Cbf1–Met4–Met28 complex is formed over the TCACGTG sequence. The experiments also show that both Met4 and Met28 bind to DNA only in the presence of Cbf1. Moreover, Met28 is shown to enhance the DNA-binding activity of Cbf1. Analysis of *MET28* gene regulation reveals that its expression requires Met4. Thus the biochemical activity of Met28 allows the establishment of a positive regulatory loop. The results thus provide evidence of a new functional relationship between bHLH and bZIP proteins and demonstrate that the association of such factors may serve to discriminate between the different TCACGTG sequences found in the chromosomes.

Keywords: bHLH–bZIP heteromeric complex/sulfur amino acid metabolism/transcriptional activation

Introduction

Considerable evidence has been accumulated showing that numerous DNA transactions, including transcription, replication, recombination, repair and chromosome segregation involve the assembly of specific multi-protein complexes (Lechner and Carbon, 1991; Foss *et al.*, 1993; Svejstrup *et al.*, 1996). Such heteromeric complexes were particularly evident in the study of core promoters of eucaryotic genes which are recognized by a set of universal proteins assembled in several complexes constituting the general transcription apparatus (Burley and Roeder, 1996; Goodrich *et al.*, 1996; Wilson *et al.*, 1996). In addition to the general apparatus, gene expression is controlled by regulatory proteins that bind to specific DNA sequences located near to or at a great distance upstream or down-

stream from the core promoter and which influence the initiation rate of transcription.

In yeast, genetic studies have drawn a general picture of regulatory proteins acting as single molecules (Struhl, 1995). In a few cases, however, transcriptional regulation was shown to be driven through the assembly of highly specific multi-component complexes. One of the best characterized examples of a transcription activation complex is the Hap2/3/4/5 complex evidenced by Guarente and his collaborators. The Hap2/3/4/5 complex binds to the DNA sequence known as the 'CCAAT' box and is involved in the transcriptional activation of numerous nuclear genes, especially those encoding mitochondrial proteins (McNabb *et al.*, 1995). The second transcription activation complex described in yeast is the Swi4–Swi6 complex which participates in the mating type switching control and also activates a number of genes at the G1–S boundary (Primig *et al.*, 1992).

We recently have described a novel heteromeric transcription activation complex in yeast which participates in the transcriptional regulation of sulfur amino acid metabolism. Using yeast cell extracts, we indeed demonstrated by gel retardation assays that the *MET16* 5' upstream region was recognized by a high molecular weight complex which was shown to contain at least three proteins, centromere-binding factor 1 (Cbf1), Met4 and Met28 (Kuras *et al.*, 1996). The Cbf1–Met4–Met28 complex exhibits several interesting features. To our knowledge, it constitutes the first instance of two basic leucine zipper (bZIP) factors, Met4 and Met28, associating together with a basic helix–loop–helix (bHLH) factor, Cbf1. Moreover, the Cbf1–Met4–Met28 complex appears to be composed of two specific transcription factors, Met4 and Met28, and one multi-functional factor, Cbf1, which is involved in both regulation of sulfur amino acid metabolism and chromosome stability (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1991). Indeed, Cbf1 binds to the CDE1 sequence, one of the three DNA elements which constitute the centromeres of *Saccharomyces cerevisiae* chromosomes (Hieter *et al.*, 1985).

First analyses of the Cbf1–Met4–Met28 complex revealed that it contains only one transcription activation module. Indeed, neither Cbf1 nor Met28 have an intrinsic transcription activation function (Thomas *et al.*, 1992; Kuras *et al.*, 1996). The only transcription activation function found so far within this complex is provided by the Met4 component. Met4 was shown to contain a unique activation domain located in the N-terminal part of the protein and whose function is controlled by the level of intracellular S-adenosylmethionine (AdoMet), the end product of the sulfur amino acid biosynthesis pathway (Kuras and Thomas, 1995a). Thus, it appears that a functional role could be ascribed to only two subunits of the Cbf1–Met4–Met28 complex: DNA recognition and

binding for Cbf1 and transcriptional activation for Met4. In contrast, the function of Met28 in this complex remained obscure: in addition to being devoid of transcription activation ability, Met28 was shown to bind by itself to DNA with a very low affinity. Its binding to DNA with a high affinity was shown to require the presence of both the Cbf1 and Met4 proteins (Kuras *et al.*, 1996).

To understand better the precise function of Met28 and to address the question of whether the assembly of the Cbf1–Met4–Met28 complex on DNA may require additional factors, we performed electrophoretic mobility shift assays and DNase I footprint experiments using recombinant Cbf1, Met4 and Met28 proteins which were expressed in *Escherichia coli* and subsequently purified. We report here the *in vitro* reconstitution of the Cbf1–Met4–Met28 complex. The set of experiments allowed us to demonstrate that Met28 functions by stimulating the DNA-binding activity of Cbf1. Moreover, the regulation of the *MET28* gene was studied and was shown to depend on Met4. Used together, the regulation of the *MET28* gene and the biochemical activity of its encoded product thus allow the establishment of a positive regulatory loop. The results exemplify the means by which eucaryotic gene regulation depends on multi-subunit protein complexes and on accessory transcription factors such as Met28.

Results

To investigate the mechanisms underlying the assembly of the Cbf1–Met4–Met28 complex and to understand better the function of Met28, different derivatives of Cbf1, Met4 and Met28 were expressed in *E.coli* as histidine-tagged proteins and subsequently purified by affinity chromatography on agarose–nickel columns (see Materials and methods). All derivatives contain the domains identified by the ‘two-hybrid’ method as providing the protein surfaces allowing the Met4–Met28 and Met4–Cbf1 interactions (Kuras *et al.*, 1996). For Cbf1, we used a derivative corresponding to residues 210–351, the expression of which was shown to be sufficient to relieve the methionine auxotrophy of a *cbf1*-disrupted strain (Mellor *et al.*, 1990).

Reconstitution of the Cbf1–Met4–Met28 complex on the *MET16*_{UAS}

Electrophoretic mobility shift assays were performed using the Cbf1, Met4 and Met28 recombinant derivatives and a DNA oligonucleotide containing the TCACGTG core sequence of the *MET16* 5' upstream region (*MET16*_{UAS}; O'Connell *et al.*, 1995) and corresponding to nucleotides –155 to –194 (numbered relative to the initiation codon). We first found that, as previously noted for the GST–Met28 fusion protein, the histidine-tagged Met28 protein bound to the *MET16* probe only in the absence of poly(dI–dC)–(dI–dC) competitor DNA (data not shown). We next examined the binding of Cbf1 to the *MET16*_{UAS} in the absence or presence of Met4 and/or Met28. As expected, recombinant Cbf1 was capable of binding to the *MET16*_{UAS} (complex I). However, when the DNA-binding reactions were performed at Cbf1 concentrations that resulted in a very low level of DNA-binding activity (Figure 1A, lanes 2–5), the addition of Met28 increased the DNA binding of Cbf1 (Figure 1A, lanes 7–10). By contrast, the addition of Met4 had little or no effect on Cbf1 activity (Figure 1A,

lanes 12–15). Thus, the enhancement of Cbf1 binding activity appears to be specific to Met28. It is important to note that this stimulation was observed without a change in the mobility of the Cbf1–*MET16*_{UAS} complex. Such a result could be accounted for by the fact that a Cbf1–Met28–DNA ternary complex was initially formed but that Met28 dissociated during electrophoresis. A similar observation has already been reported for the stimulation of the DNA-binding activity of the serum response factor (SRF) by the homeodomain protein Phox (Gruneberg *et al.*, 1992), as well as for the induction of DNA binding of several bZIP factors by the viral protein Tax (Wagner and Green, 1993). When Cbf1, Met4 and Met28 were added together to the DNA-binding assays, two complexes were formed (Figure 1A, lanes 16–20): they correspond to complex I and to a complex of slower electrophoretic mobility (complex II).

Since complex II was only observed when the three recombinant proteins factors were added to the DNA-binding reactions, it is likely that it corresponds to the assembly of the Cbf1–Met4–Met28 complex on the *MET16*_{UAS}. Furthermore, the fact that the amounts of complexes I and II are similar suggests that Met28 exerts its stimulatory effect on both complexes and therefore that they both contain Cbf1.

To confirm these observations further, we performed two additional series of mobility shift assays. In the first one, increasing concentrations of Met4 were incubated in the presence of high concentrations of Cbf1 and/or Met28. As shown in Figure 1B, no complex corresponding to the binding of Met4, alone or together with Cbf1 or Met28, could be observed [lanes 2–12; the slow mobility complex observed with high concentrations of Cbf1 may result from the multimerization of Cbf1 on DNA as previously observed for several bHLH factors (Ferré d'Amaré *et al.*, 1994)]. As expected, complex II formed when the three proteins were mixed in the DNA-binding reactions. It is noteworthy that, in the presence of high concentrations of both Cbf1 and Met28, the subsequent addition of Met4 lowered the amount of complex I (Figure 1B, compare lane 13 with lane 14). This can be accounted for by the displacement of Cbf1 from complex I to complex II, thus confirming that Cbf1 is present in both complexes. In a second set of experiments, we introduced size variants of Met4 or Met28 in DNA-binding assays. The use of the GST–Met28 fusion protein (395 residues) in place of the histidine-tagged Met28 recombinant protein (206 residues) decreased the mobility of complex II (Figure 2, compare lanes 3 and 4 with lane 2). In the same way, the mobility of complex II is lowered when the 6HMet4D5 protein (243 residues) was used in place of the 6HMet4-1 protein (145 residues, Figure 2, compare lanes 5 and 6 with lane 2). These results prove that complex II contains Met4 and Met28. This figure also reveals a complex of intermediate mobility (Figure 2, lanes 3 and 4) that may correspond to a Met4–Cbf1–DNA ternary complex resulting from the dissociation of Met28 during electrophoresis.

In summary, the experiments demonstrate that the assembly of the Cbf1–Met4–Met28 complex on the *MET16*_{UAS} does not require additional yeast factors and that neither Met4 nor Met28 can bind the *MET16*_{UAS} in the absence of Cbf1.

Fig. 1. Reconstitution of the Cbf1–Met4–Met28 complex on the *MET16*_{UAS}. (A) Cbf1, Met4 and Met28 recombinant proteins were assayed for DNA binding to an oligonucleotide containing the TCACGTG sequence from the *MET16* 5' upstream region. Increasing amounts (10, 20, 40 and 80 ng) of recombinant 6HCbf1ΔN were incubated with the *MET16* probe in the absence (lanes 2–5) or presence of either 60 ng of 6HMet28 (lanes 7–10), 60 ng of 6HMet4-1 (lanes 12–15) or 60 ng of both 6HMet28 and 6HMet4-1 (lanes 17–20; cxI = complex I, cxII = complex II). (B) Met4 binding to the *MET16*_{UAS} requires both Cbf1 and Met28. Increasing amounts (30, 60 and 120 ng) of 6HMet4-1 were incubated with the *MET16* probe in the absence (lanes 2–4) or presence of either 80 ng of 6HCbf1ΔN (lanes 6–8), 120 ng of 6HMet28 (lanes 10–12) or both 80 ng of 6HCbf1ΔN and 120 ng of 6HMet28 (lanes 14–16).

The Cbf1–Met4–Met28 complex extends the Cbf1-dependent DNase I protection over the *MET16*_{UAS}

Previous two-hybrid studies have shown that Met4 and Cbf1 on the one hand and Met4 and Met28 on the other hand could interact with each other (Kuras *et al.*, 1996). Together with the experiments described above, these results are consistent with the binding of the three proteins in the close vicinity of the TCACGTG sequence, the target of Cbf1. However, none of these results formally exclude the possibility that the three proteins bind to separate DNA sequences. To discriminate between the two possibilities, we performed DNase I protection experiments using a *MET16* probe corresponding to nucleotides –126 to –272.

At low concentrations of Cbf1, footprints over the TCACGTG sequence were only observed in the presence of Met28 (Figure 3A and B, compare lane 2 with lanes 6 and 7). At high concentrations of Cbf1, the footprints were identical on both strands to those observed when Met28 was mixed with small amounts of Cbf1 (Figure 3A and B, compare lanes 3 and 4 with 7 and 8). Thus, in both mobility shift analyses and footprint assays, Met28

enhances the binding of Cbf1 to the TCACGTG sequence. In contrast, the presence of Met4 did not modify the Cbf1 footprint (Figure 3A and B, compare lanes 3 and 12).

In the absence of Cbf1, addition of Met4 and/or Met28 to the DNA-binding reactions did not lead to a footprint (Figure 3A and B, lanes 5, 9 and 13). In the presence of Cbf1, the addition of Met4 together with Met28 to the DNA-binding assays enhanced the DNase I protection activity and, moreover, extended the protected region on both strands (Figure 3A and B, lanes 14–16). It must also be noted that the addition of the three proteins Cbf1, Met4 and Met28 led to hypersensitive sites outside of the protected site, suggesting that the assembly of the Cbf1–Met4–Met28 complex may have a long range effect on the DNA structure. A summary of the data obtained is presented in Figure 3C, showing that the assembly of the Cbf1–Met4–Met28 complex extends the DNase I footprint mainly toward the 5' extremity of the *MET16*_{UAS}. Furthermore, the experiments confirmed that the Cbf1–Met4–Met28 complex is assembled on the TCACGTG core sequence.

Fig. 2. Complex II contains Met4 and Met28. Size variants of Met4 and Met28 were incubated with the *MET16* probe and 6HCbf1 Δ N. DNA-binding assays contained 20 ng of 6HCbf1 Δ N, 60 ng of 6HMet28 and 60 ng of 6HMet4-1, except lanes 3 and 4 which contained 30 and 60 ng respectively of GST-Met28 in place of 6HMet28 and lanes 5 and 6 that contained 30 and 60 ng respectively of 6HMet4-5 in place of 6HMet4-1.

Met28 stimulation of Cbf1 DNA binding

In addition to demonstrating that the Cbf1-Met4-Met28-*MET16*_{UAS} complex could be reconstituted *in vitro*, the experiments reported above also revealed that Met28 enhances the DNA-binding activity of Cbf1. To establish that this effect was due to an intrinsic activity of the Met28 protein and not to the additional N-terminal residues found in the histidine-tagged derivative, we used the recombinant GST-Met28 fusion protein (Kuras *et al.*, 1996). As shown in Figure 4A, incubations of a small amount of Cbf1 with increasing amounts of the GST-Met28 recombinant protein in DNA-binding assays resulted in increased amounts of complex I (maximum enhancement was reached at 60 ng of added GST-Met28 protein). Thus, the effect of Met28 is likely to reflect an authentic activity of the protein.

Like several bHLH factors, Cbf1 is known to bind DNA as a dimer (Dowell *et al.*, 1992). Met28 could therefore stimulate DNA binding of Cbf1 by increasing either the dimerization or the subsequent interactions with DNA. The former possibility is unlikely since Cbf1 dimers are known to be extremely stable in solution. In agreement with this, in the presence as well as in the absence of Met28, binding of Cbf1 heterodimers to the *MET16*_{UAS} was not observed in mobility shift assays after both a full-length and a truncated histidine-tagged Cbf1 derivative were mixed in DNA-binding assays (data not shown). We next examined the association and dissociation rates of Cbf1-DNA complexes in the presence or absence of Met28. In the absence of Met28, binding of Cbf1 to DNA reached a maximal level only 1 min after the protein was mixed with DNA (Figure 4B). The same result was obtained for all Cbf1 concentrations, in the absence or presence of Met28 (data not shown). Thus, the Cbf1-DNA complex formed so rapidly that, if it exists, a Met28-mediated acceleration rate of Cbf1-DNA complex formation cannot be tested in mobility shift assays. To

determine the dissociation rates of the Cbf1-*MET16*_{UAS} complex, DNA binding was allowed to reach equilibrium, excess unlabelled specific competitor DNA was added and the amount of remaining Cbf1-*MET16*_{UAS} was measured as a function of time. As shown in Figure 4C, the presence of Met28 lowered the dissociation rate of the Cbf1-DNA complex. We thus conclude that the Met28 enhancement of Cbf1 DNA-binding activity can be, in part, accounted for by a decrease in the dissociation rate of the Cbf1-DNA complex.

Regulation of MET28 gene transcription

Taken together, the results reported above and those previously reported (Kuras *et al.*, 1996), demonstrate that Met28 mainly functions by stimulating the assembly of a transcription activation complex on the TCACGTG core sequence present in front of several *MET* genes. To understand how such a biochemical activity could be used by yeast cells to regulate the metabolic flux between sulfate and AdoMet, we decided to investigate the regulation of the *MET28* gene itself. We thus examined whether its expression may be regulated by the level of intracellular AdoMet, as previously observed for all the structural genes of sulfur amino acid metabolism (Thomas *et al.*, 1989). Transcriptional regulation of the *MET28* gene was monitored by shifting wild-type cells grown in a medium containing a non-repressing amount of methionine (0.05 mM L-Met) to one containing a repressing amount (1 mM L-Met). Total RNA was then extracted at regular intervals after the shift and analysed with radiolabelled probes specific for the *MET4*, *MET25* and *MET28* genes. As previously reported (Thomas *et al.*, 1995), the addition of a repressing amount of methionine to the medium repressed the transcription of *MET25* whose mRNAs were undetectable 20 min after the shift. (Figure 5A). In contrast, the level of *MET4* mRNAs remained unchanged, showing that this gene is constitutively transcribed. For the *MET28* gene, a rapid decrease in its mRNAs was measured after addition of methionine: *MET28* transcripts were undetectable 5 min after the shift. The transcription of the *MET28* gene thus appears to be repressed in response to an increase in intracellular AdoMet. As shown in Figure 5A, analysis of total RNA extracted each minute after methionine addition confirmed that *MET28* mRNAs rapidly disappeared, being virtually undetectable 4 min after the shift.

The *MET25* and *MET28* transcript decay rates thus differ significantly when yeast cells are shifted to repressive growth conditions. Such a result may arise from a different stability of each transcript or from a specific repression mechanism acting on *MET28* gene expression. To discriminate between the two possibilities, we measured the turnover of each RNA by using a *rpb1-1* mutated strain. The *rpb1-1* mutation is a temperature-sensitive lesion in the largest subunit of RNA polymerase II (Nonet *et al.*, 1987). A shift of *rpb1-1* cells to a non-permissive growth temperature (36°C) leads to a rapid arrest of mRNA synthesis, and Northern analyses of ongoing mRNAs thus allow precise measurement of the turnover of individual mRNAs (Herrick *et al.*, 1990). The decay rates of *MET25* and *MET28* mRNAs after the temperature shift of *rpb1-1* cells are illustrated in Figure 5B. The *MET28* mRNAs decayed more rapidly than the *MET25* mRNAs, with half-lives of <5 min and ~15 min, respectively. These

Fig. 3. DNase I footprint assays of Cbf1, Met28 and Met4. (A) and (B) show the upper and the lower strands, respectively. Binding reactions contained Cbf1 alone (40, 80 and 160 ng, lanes 2–4), lower concentrations of Cbf1 (20, 40 and 80 ng) and either 120 ng of 6HMet28 (lanes 6–8), 120 ng of 6HMet4-1 (lanes 10–12) or 120 ng of both 6HMet28 and 6HMet4-1 (lanes 14–16). Lanes 5, 9 and 13 show the footprints obtained with 120 ng of either 6HMet28 or 6HMet4-1 or both 6HMet28 and 6HMet4-1. Lanes 1 and 17 show protein-free ladders. The solid bars mark the footprints observed at high concentrations of Cbf1 alone or at low concentrations of Cbf1 in the presence of Met28 (light grey) and the footprint observed when Cbf1, Met4 and Met28 were added to the binding reactions (dark grey). (C) The data in (A) and (B) are summarized, with light grey and dark grey boxes marking the footprints obtained with either Cbf1 alone or Cbf1 and Met28, and with Cbf1, Met4 and Met28, respectively. The corresponding hypersensitive sites are indicated by light and dark grey ovals. The TCACGTG core sequence at position –175 in the *MET16* 5' upstream region is in white.

experiments demonstrate that the different decay rates measured for the *MET28* and *MET25* transcripts after the methionine repressing shift were actually due to their different stability. Taken together, the two sets of Northern experiments also suggest that the addition of 1 mM of L-methionine to the growth medium leads to a rapid and concerted cessation of transcription initiation events at the *MET* promoters.

***MET28* transcription activation depends on Met4**

The AdoMet-mediated repression of *MET28* gene expression prompted us to test whether its transcriptional activation might require the different components of the Cbf1-Met4-Met28 complex. Such a hypothesis was reinforced by the presence of a TCACGTG sequence in the 5' upstream region of the *MET28* gene (at position –280). We thus compared the transcription of the *MET28* gene

Fig. 4. (A) Stimulation of the DNA-binding activity of the 6HCbf1ΔN derivative by the recombinant GST-Met28 protein. Binding assays contained 20 ng of 6HCbf1ΔN and increasing concentrations of GST-Met28 (lanes 2–7: 0, 15, 30, 60 and 120 ng of GST-Met28 respectively). (B) Association rate of Cbf1 with the *MET16*_{UAS}. Sixty ng of 6HCbf1ΔN was incubated at 4°C with the *MET16* probe for the indicated times, and binding reactions were loaded immediately on a running gel. (C) Dissociation rates of Cbf1 in the absence and presence of Met28. Cbf1–*MET16*_{UAS} complexes were allowed to form for 15 min at 4°C in the absence (lanes 2–5) and presence (lanes 6–9) of 6HMet28 and then a 100-fold excess of unlabelled *MET16*_{UAS} competitor was added for the indicated times before reactions were loaded on the running gel.

in *cbf1* and *met4* mutant cells with that measured in wild-type cells. This was done by shifting the different strains from a medium containing a repressing amount of methionine to one without methionine and then extracting the

RNAs at regular time intervals. Total RNAs were analysed with a *MET28* probe. As expected from the above results, derepression of *MET28* transcription is measured: *MET28* mRNAs are first detected 20 min after the shift and transcription reaches its maximal level 80–100 min after the shift (Figure 5C). The kinetics of *MET28* derepression thus appear to be very similar to those of the methionine biosynthetic genes (Kuras and Thomas, 1995b). Furthermore, this set of experiments revealed that *MET28* transcription strictly depends on the integrity of Met4: *MET28* transcripts could not be detected in *met4*-disrupted cells. In contrast, the *cbf1* disruption mutation weakly affects the expression of *MET28* by increasing the time needed for the transcription to reach its maximum level. To confirm these results and to analyse the effect of the *met28* mutation on its own promoter region, *met4*, *met28* and *cbf1* mutant strains were transformed by a *MET25-lacZ* or a *MET28-lacZ* reporter gene, and β-galactosidase activities were assayed after the transformed cells were grown in both non-repressive and repressive conditions. The data (Figure 6A) confirmed that Met4 is involved in transcription activation of the *MET28* gene: the *MET28-lacZ* reporter gene activity is 17-fold lower in a *met4*-disrupted strain than in a wild-type strain. As expected from Northern analyses (Kuras and Thomas, 1995b; Kuras *et al.*, 1996; this study), in *cbf1* mutant cells, but not in *met28* mutant cells, the activity of the *MET25-lacZ* gene fusion is 2-fold less than that measured in wild-type cells. On the other hand, the activity of the *MET28-lacZ* gene fusion is only 2-fold lowered in both *cbf1* and *met28* mutant cells.

Purified Cbf1, Met4 and Met28 proteins do not form a complex on the *MET28* 5' upstream region

The regulation of *MET28* gene expression provides a new display of the diversity of molecular mechanisms sustaining the metabolic control of the sulfate assimilation pathway. As shown above, like all of the structural genes of this pathway, the activation of *MET28* transcription is indeed strictly dependent on an active Met4 protein (Thomas *et al.*, 1990, 1992). However, unlike the *MET10*, *MET14* and *MET16* genes whose transcriptional activation is also dependent on Cbf1 and Met28 proteins (Kuras and Thomas, 1995b; Kuras *et al.*, 1996), *MET28* expression is only weakly affected by mutations inactivating the two factors. This result is reminiscent of that obtained for the *MET3* and *MET25* structural genes whose expression is independent, respectively, of the Cbf1 and Met28 proteins. These results prompted us to determine whether the Cbf1–Met4–Met28 complex could be formed on the 5' upstream region of *MET28*. We thus performed mobility shift assays with a probe specific for the *MET28* gene and Cbf1, Met4 and Met28 recombinant proteins. The *MET28* probe corresponds to nucleotides –305 to –260 and contains the CACGTG sequence found at position –280. As shown in Figure 6B, Cbf1 is indeed capable of binding to the *MET28* probe and, as for the *MET16* probe, its binding activity is enhanced by the addition of the Met28 protein. However, the combination of Met28 and Met4 together with Cbf1 did not result in a complex of slow mobility corresponding to the assembly of the Cbf1–Met4–Met28 complex on the *MET28* probe. It must be noted further

Fig. 5. (A) *MET28* transcription is repressed by an increase of intracellular AdoMet. A wild-type strain (W303-1A) was grown in B medium (Cherest and Surdin-Kerjan, 1992) in the presence of 0.1 mM sulfate as sulfur source. When the cell concentration reached $\sim 10^7$ /ml, L-methionine was added to the medium at a final concentration of 1 mM. Samples were then withdrawn at the indicated times and total RNA was extracted. For each time point, 10 μ g of total RNA was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. The transferred RNAs were hybridized with probes specific to the *MET4*, *MET25* and *MET28* genes. The actin probe was used as a control for the amounts of RNA loaded. (B) *MET28* mRNA decay rate. Y260 cells (*rpb1-1*) were grown at 24°C and shifted abruptly to 36°C by adding an equal volume of minimal medium at 48°C and then transferring the culture flask to a shaker bath at 36°C. Total RNA was then extracted at the indicated times. Ten μ g of total RNA were electrophoresed, transferred onto a nylon membrane and hybridized with probes specific to the *MET25*, *MET28* and actin genes. (C) *MET28* transcription activation depends on Met4. Derepression kinetics of *MET28* transcription were monitored in wild-type cells and in cells lacking either Met4 or Cbf1. The cells used were the strains W303-1A (wild-type), CC718-1A (*cbf1::TRP1*) and CD106 (*met4::TRP1*). They were grown in B medium in the presence of a repressing amount (1 mM) of L-methionine as sulfur source. When the cells reached a density of $\sim 10^7$ cells/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, electrophoresed, transferred onto a nylon membrane and hybridized to a *MET28*-specific probe. The actin probe was used as a control for the amounts of RNA loaded.

that, on a *MET28* probe, the DNA-binding activity of Cbf1 is also enhanced by the Met4 derivative.

Discussion

Involvement of the CACGTG hexameric motif as a *cis*-acting element in many DNA transactions appears to be widely distributed through all of the eucaryotic life kingdom. As a consequence, this short sequence has been given various names, depending on the organism. In mammals, the CACGTG sequence is called the E-element and is recognized by different DNA-binding proteins, including the ubiquitous factor USF as well as several

members of both the MyoD and Myc families of mammalian proteins (Blackwell *et al.*, 1990; Gregor *et al.*, 1990; Blackwood and Eisenman, 1991). The E-element thus plays a major role in various pathways of cellular regulation as different as glucose responsiveness and myogenic differentiation (Murre *et al.*, 1989; Lefrancois-Martinez *et al.*, 1995). In plants, the CACGTG sequence is known as the G-box and has been shown to be essential for the function of many promoters (Donald *et al.*, 1990). The plant G-box is thus involved in the responsiveness of several genes to different stimuli including light, anaerobiosis and several vegetal hormones (Menkens *et al.*, 1995). Most of the plant G-box-binding proteins belong

Fig. 6. (A) Effect of Met28 on its own promoter. Wild-type cells as well as those lacking either Cbf1, Met4 or Met28 were transformed by a multi-copy plasmid containing either a *MET28-lacZ* or a *MET25-lacZ* reporter gene. The β -galactosidase activities were measured with cells grown in non-repressive (0.05 mM L-methionine, grey bars) and repressive (1 mM L-methionine, black bars) growth conditions. The reported values represent an average of at least three assays performed with independent transformants and were expressed as nanomoles of substrate transformed/min/mg of protein. Standard deviations were <15%. (B) Mobility shift assays of Cbf1, Met4 and Met28 recombinant proteins on a *MET28* probe. Cbf1, Met4 and Met28 recombinant proteins were assayed for DNA binding to an oligonucleotide containing the TCACGTG sequence from the *MET28* 5' upstream region. Forty ng of recombinant 6HCbf1ΔN was incubated with the *MET28* probe in the absence (lane 2) and presence of either 80 ng of 6HMet28 (lane 3), 80 ng of 6HMet4-1 (lane 4) or 80 ng of both 6HMet28 and 6HMet4-1 (lane 5)

to the family of bZIP factors (Schindler *et al.*, 1992). In yeast, the CACGTG sequence was first called CDE1 (for centromere determining element 1) and was known to play a major role in three different cellular 'mechanisms': chromosome segregation, phosphate metabolism and sulfur amino acid biosynthesis (Hieter *et al.*, 1985; Rudolph and Hinnen, 1987; Thomas *et al.*, 1989). With regard to its ubiquitous function, it is likely that the functional specificity of this *cis*-acting sequence is achieved through both sequence elements in the vicinity of the core CACGTG motif and different protein-protein interactions.

Saccharomyces cerevisiae is a particularly propitious organism with which to question the CACGTG functional specificity since, in addition to being a genetically tractable organism, thus allowing *in vivo* studies, yeast cells contain two bHLH factors, Pho4 and Cbf1, which bind this motif (Lemire *et al.*, 1985; Baker *et al.*, 1989). From previous *in vitro* studies, it is believed that the systematic presence of a 5' T residue flanking the CACGTG sequence in both

the CDE1 element of the centromeres and the regulatory region of the methionine biosynthetic genes constitutes a major specificity determinant by preventing the binding of Pho4 (Fisher and Goding, 1992). Accordingly, inactivation of the *CBF1* gene results both in altered expression of several methionine biosynthetic genes and in a decreased fidelity of chromosomal segregation, but is without effect on phosphate metabolism (Cai and Davis, 1990; Mellor *et al.*, 1990; Thomas *et al.*, 1992). Nevertheless, how the bi-functionality of the TCACGTG-Cbf1 pair is gained remained a question for discussion.

In a previous study, we have demonstrated that the *MET16* 5' upstream region is recognized by a high molecular weight complex. By using extracts of cells lacking either the Cbf1 or Met4 or Met28 proteins, we showed that this complex contained these three factors (Kuras *et al.*, 1996). The present study deals with the assembly of this complex. Using purified recombinant proteins, we show here that the Cbf1-Met4-Met28 complex can form on the *MET16*_{UAS} without any additional yeast factors. Furthermore, DNase I footprint assays revealed that the Cbf1-Met4-Met28 complex is formed over the TCACGTG core sequence. Actually, assembly of the Cbf1-Met4-Met28 complex extends the DNase I-protected region on 6–7 nucleotides 5' adjacent to the TCACGTG sequence. To our knowledge, this constitutes the first demonstration that two bZIP factors and one bHLH factor can associate together to bind a specific DNA target. Taken together with the previous two-hybrid studies revealing that the leucine zippers of Met4 and Met28 along with the bHLH domain of Cbf1 provide the protein surfaces mediating the assembly of the Cbf1-Met4-Met28 complex (Kuras *et al.*, 1996), the results of the DNase I protection experiments provide evidence that combination of two bZIP and a bHLH domain can form a particular DNA-protein interaction surface, thus extending the DNA area recognized by bHLH factors. The Cbf1-Met4-Met28 complex thus provides a good example of how different protein-protein interactions may serve to discriminate between the different TCACGTG sequences found in the chromosomes. Although we do not know whether the Cbf1-Met4-Met28 complex may be assembled or not on yeast centromeres, from our results it can be postulated that differences in the nucleotides 5' adjacent to the TCACGTG core sequences would constitute the sequence determinants that prevent the formation of a transcription activation complex on the centromeres.

Results presented here revealed the functional role of the Met28 protein in the TCACGTG-binding complex. Through the use of LexA-Met28 fusion proteins, we have demonstrated previously that Met28 does not possess an intrinsic transcription activation function (Kuras *et al.*, 1996). Furthermore, as both the GST-Met28 and 6HMet28 recombinant proteins were shown to bind DNA with a low affinity, the reasons why the *met28* disruption mutation impairs the transcription activation of several methionine genes remained undeciphered. The results reported here clearly show that the function of Met28 is dedicated to the assembly of the Cbf1-Met4-Met28 complex in two different ways: (i) in the absence of Met28, in both mobility shift assays and DNase I footprint experiments, Met4 cannot bind to the *MET16*_{UAS} even in the presence of Cbf1, although previous two-hybrid studies revealed a

direct interaction between the bZIP domain of Met4 and the bHLH domain of Cbf1; (ii) both mobility shift assays and DNase I protection experiments demonstrate that the Met28 protein is endowed with a biochemical activity which allows it to stimulate the DNA-binding activities of Cbf1 and, therefore, of the Cbf1–Met4–Met28 complex. These results thus reveal a novel functional relationship between two classes of widely distributed DNA-binding proteins, the bZIP and bHLH factors. We can predict that the possibility of a bZIP factor specifically to enhance the DNA-binding activity of a bHLH factor should constitute a general property of such proteins. For instance, it was shown that auto-regulation of the human gene encoding the bZIP CEBP/α factor arises through the stimulation of the DNA-binding activity of the bHLH USF factor (Timchenko *et al.*, 1995). However, the conclusions were reached from transient infection experiments without demonstration of a direct interaction between CEBP/α and USF. It is noteworthy that the members of the CEBP/α class are the bZIP factors which are the most closely related to the Met28 protein, in having an extra seven amino acid segment between the basic domain and the leucine zipper (Landschulz *et al.*, 1988), and that USF is the mammalian counterpart of the Cbf1 protein.

In yeast, the functional relevance of the Met28 stimulatory effect on the DNA-binding activity of Cbf1 is sustained by the analysis of *MET28* gene regulation. Indeed we show here that transcription of the *MET28* gene is repressed specifically by the increase in intracellular AdoMet. Moreover, the activation of *MET28* transcription appears to depend on a functional Met4 protein. Thus the biochemical activity of the Met28 protein is taken into account, allowing the construction of a positive regulatory loop (Figure 7): the Met28 protein allows the binding of the Met4 protein to the *MET28* 5' upstream region with a high affinity through the assembly of the Cbf1–Met4–Met28 complex while the Met4 protein allows the transcription of the *MET28* encoding gene. Such a regulatory loop may thus permit fine regulation of the metabolic flux between sulfate and AdoMet. At high intracellular concentrations of AdoMet, it is known that the transcription activation function of Met4 is inhibited by the Met30 protein, a transcriptional inhibitor which was shown to interact specifically with Met4 (Thomas *et al.*, 1995). The speculative model drawn in Figure 7 also predicts the involvement of an additional and to date unknown DNA-binding factor. Indeed, both Northern analyses and *MET28*–*lacZ* gene fusion experiments suggest that, in contrast to Met4, the Cbf1 and Met28 proteins have only an accessory function in transcriptional activation of the *MET28* gene. Since we show here that Met4 is not able to bind either by itself or together with Cbf1 and Met28 to the *MET28* 5' upstream region, the presence of an additional factor responsible for Met4 binding therefore appears to be required to explain the Met4-dependent transcriptional activation of the *MET28* gene. This model would suggest that the Cbf1–Met4–Met28 complex actually represents a particular class of a complex family whose different members may recognize promoter regions of different structural genes from sulfur amino acid metabolism in order to achieve the coordinated regulation of their expression. Such a hypothesis would explain why the different structural genes involved in this metabolism,

Fig. 7. Model for the regulatory loop implicated in the regulation of the metabolism of sulfur amino acids. Met30 is the AdoMet-responsive transcription inhibitor which was shown to interact with Met4 and to control its transcription activation function (Thomas *et al.*, 1995). pX represents the postulated DNA-binding factor required for the targeting of Met4 to the *MET28* promoter.

as well as the *MET28* gene itself, are affected differently by the *cbf1* and *met28* disruption mutations. It thus appears that, even for a simple gene network, the assembly of different combinations of multi-subunit complexes on regulatory sequences may be required to achieve the correct control of a metabolic flux.

Materials and methods

Yeast strains and media

The *S.cerevisiae* strains used in this work were: W303-1A (*Mata*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*); CC718-1A (*Mata*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *cbf1::TRP1*); CC767-6A (*ade2*, *his3*, *leu2*, *trp1*, *ura3*, *met28::URA3*); CD106 (*Mata*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *met4::TRP1*) (Kuras *et al.*, 1996); and Y260 (*MATa*, *ura3*, *rpb1-1*) (Nonet *et al.*, 1987). Standard yeast media were prepared as described by Cherest and Surdin-Kerjan (1992). *S.cerevisiae* were transformed after lithium acetate treatment as described by Gietz *et al.* (1992).

Plasmid constructions

The set of pET28 vectors (Novagen, Madison, WI) were used to produce the histidine-tagged recombinant proteins. The 6HCbf1ΔN derivative contains residues 210–351 of Cbf1 and was constructed by transferring an *EcoRI*–*Sall* fragment from pLexCBF1 (Thomas *et al.*, 1992) into pET28a. The 6HMet28 derivative contains all the Met28 residues and was constructed by cloning an *EcoRI*–*Sall* fragment from pLexM28-2 (Kuras *et al.*, 1996) into pET28a. The 6HMet4-1 derivative contains the Met4 residues 559–666 and was constructed by transferring a *Sall*–*Sall* fragment of pM4-4 (Thomas *et al.*, 1992) into pET28b. The 6HMet4Δ5 derivative contains the Met4 residues 15–56 and 501–666, and was constructed by transferring an *EcoRI*–*BamHI* fragment of pLexM4Δ5 (Kuras and Thomas, 1995a) into pET28b.

Protein purifications

Bacterially synthesized histidine-tagged recombinant Cbf1, Met4 and Met28 proteins were purified by affinity chromatography on nickel–agarose columns. For the 6HMet28 derivative, BL21(ΔDE3) *E.coli* cells harbouring the pETMet28 plasmid were grown at 37°C in 1 l of L broth

containing 50 µg/ml kanamycin to an $OD_{650\text{ nm}} = 1$. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and growth was continued for another 4 h at 37°C. The following procedures were performed at 4°C with ice-cold buffers. Cells were harvested by centrifugation, resuspended in one volume of buffer A [20 mM Tris-HCl (pH 8.0), 1 M NaCl, 5 mM imidazole, 10% glycerol] containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the cells were lysed by passing the suspension through an Eaton press. The cell extract was diluted with one volume of buffer A containing 1 mM PMSF, 10 mg/ml RNase A and 5 mg/ml DNase I and incubated for 15 min in ice. Insoluble material was removed by centrifugation at 30 000 g for 30 min. The clear lysate was then loaded onto a 4 ml Ni^{2+} -NTA-agarose column (Qiagen, Hilden, Germany) and chromatography was performed at a flow rate of 0.5 ml/min. The loaded column was washed with 40 ml of buffer A and then with 120 ml of buffer B [20 mM Tris-HCl (pH 8.0), 1 M NaCl, 60 mM imidazole, 10% glycerol]. Bound proteins were eluted with buffer C [20 mM Tris-HCl (pH 8.0), 1 M NaCl, 340 mM imidazole, 10% glycerol]. The fractions containing 6HMet28 proteins were aliquoted and stored at -80°C. For the purifications of the 6HMet4-1 and 6HMet4ΔN derivatives, the same procedure was applied except that buffers contained 500 mM NaCl. For the purification of the 6HCbf1 derivatives, the buffers were: A, 50 mM Na_2HPO_4 (pH 7.5), 300 mM NaCl, 10% glycerol; B, 50 mM Na_2HPO_4 (pH 7.5), 300 mM NaCl, 40 mM imidazole, 10% glycerol; C, 50 mM Na_2HPO_4 (pH 7.5), 300 mM NaCl, 250 mM imidazole, 10% glycerol. Protein concentrations were determined using the Bradford assay.

Electrophoretic mobility shift assays

All mobility shift assays were performed in 20 µl of HDB buffer (25 mM HEPES pH 7.6, 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$) and 0.5 mg/ml bovine serum albumin (BSA), 7.5% glycerol, 0.8 µg of poly(dI-dC)-poly(dI-dC) competitor DNA with various amounts of recombinant proteins as indicated in the figure legends. Oligonucleotide probes were 5' labelled by using T4 DNA kinase and [γ - ^{32}P]ATP (3000 Ci/mmol; Amersham). Approximately 20 000 c.p.m. of probe (~0.25 ng) was used in each binding mixture. Samples were incubated for 15 min in 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 9 V/cm at 7°C. Gels were pre-electrophoresed for 45 min at 5 V/cm at 7°C. Gels were run for 2 h, dried and autoradiographed for 15 h with an intensifying screen. For the gel shift mobility assays on the MET16 probe, we used the oligonucleotide

5' ATTTTATCATCATTTACGCTGGCTAGTAAAAGAAAGCC 3'
3' TAAAAATAGTAGTAAGTGCACCGATCATTTTCTTTTCGG 5'

corresponding to nucleotides -155 to -194 of the MET16 5' upstream region (numbered relative to the initiation codon).

DNase I footprinting assays

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

Northern blot analyses

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

mRNA decay analyses

mRNA decay rates were measured as described by Herrick *et al.* (1990) with cultures of strain Y260 which carries a *rpb1-1* mutation, a thermosensitive lesion within the largest subunit of RNA polymerase II. One hundred ml of Y260 cells (1×10^7 cells/ml) grown at 24°C were abruptly shifted to 36°C by adding an equal volume of minimal medium at 48°C and then transferring the culture flask to a shaker bath at 36°C. Total RNA was then extracted at regular time intervals after the shift and analysed by Northern blotting.

β -Galactosidase assays

β -Galactosidase assays were performed as described by Kuras and Thomas (1995a). Briefly, $\sim 10^8$ exponentially growing cells were harvested by centrifugation at 4°C and the pellet was immediately frozen in dry-ice and stored at -80°C. For the assay, the cells were washed once in 1 ml of ice-cold 100 mM Tris-HCl pH 8.0 containing 1 mM PMSF, re-suspended in 200 µl of the same buffer and then broken by vortexing 10×15 s in the presence of glass beads. Following addition of 100 µl of ice-cold 100 mM Tris-HCl pH 8.0 containing 1 mM PMSF, the extract was spun for 5 min at 4°C. β -Galactosidase activity was assayed with 5, 10, 15 and 20 µl of each extract. The protein concentration of each extract was determined by the method of Lowry *et al.* (1958). For each reporter gene, three independent transformants were assayed and the standard error was <15%.

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