Identification of the yeast methionine biosynthetic genes that require the centromere binding factor 1 for their transcriptional activation

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Abstract The yeast Centromere binding factor I (Cbf1) belongs to the family of the DNA binding factors that recognize the consensus sequence CACGTG. Phenotypic studies of cells lacking Cbf1 revealed that this factor is actually involved in two cellular processes; the fidelity of the chromosomal segregation and the metabolism of sulfur amino acids. However, the function of Cbf1 in the regulation of the sulfur amino acid metabolism is now a matter of controversy in literature with conflicting reports about its binding to the CACGTG sequences found upstream to the methionine biosynthetic genes. To provide a reliable basis for the functional analysis of Cbf1, we present an analysis of the transcription of the methionine biosynthesic genes in cells lacking Cbf1. Our results prove that Cbf1 is indeed involved in the transcriptional regulation of the sulfur amino acid metabolism.

Key words: Transcriptional regulation; Sulfur amino acid metabolism

1. Introduction

Centromere binding factor I (Cbf1, also called Cp1, Cpf1) is one of the most abundant DNA binding proteins from the yeast *Saccharomyces cerevisiae*. Cbf1 belongs to the family of the basic-Helix-Loop-Helix-Leucine Zipper (bHLH-LZ) factors that bind to the consensus sequence CACGTG [1–3]. While this sequence is largely scattered on the yeast genome (it is expected to occur ~1,700 times in the haploid yeast genome, [4]), its functional implication in a DNA process was associated with its presence in only two locations: (i) the motif CACGTG indeed constitutes the Centromere Determining Element I (CDE1) which is conserved in all the *S. cerevisiae* centromeres. Deletion of the CDE1 8-base-pair sequence results in a 10 fold increase in the frequency of mitotic chromosome loss [5–7]; (ii) the sequence CACGTG is found upstream of all the genes governing the sulfate assimilation pathway, a part of the sulfur amino acid metabolism. Mutation analyses have revealed that an intact CDE1 element is required for the transcriptional activation of two of these genes, *MET25* and *MET14* [8,9]. The Cbf1 encoding gene was isolated, allowing its disruption in haploid strains. Deletion of the *CBF1* gene results in an increase of chromosome loss rate and renders the cells unable to grow in absence of an organic sulfur source [1,2]. First analyses revealed that the requirement for Cbf1 in sulfur amino acid biosynthesis appeared to be exerted at the transcriptional level [10]. These results therefore suggested that the role of the CDE1 motif in both the chromosome segregation fidelity and the transcriptional activation of sulfur metabolism was indeed mediated through Cbf1.

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Additional evidences were later obtained strongly suggesting that Cbf1 functions in the two processes through its specific binding to the CDE1 sequence. The fact that deleting both the CDE1 sequence and the gene *CBF1* is equivalent on mitotic chromosome segregation to that of deleting either sequence alone [1] is a strong indication that Cbf1 binds to the CDE1 sequence in carrying out its role in chromosome segregation. Likewise, several lines of evidence strongly argue that Cbf1 functions in transcriptional activation through its interaction with the CDE1 sequence found in front of the sulfur genes. For instance, it is known that overexpression of Pho4, another yeast DNA binding factor which recognizes the CACGTG core motif, renders methionine prototroph the *cbf1* null mutant, providing that the strain expresses a functional Pho80 protein [11].

In spite of all this evidence, the function of Cbf1 in transcriptional activation appears to be now a matter of controversy in literature. Indeed, for several years, J. Mellor and collaborators have proposed a model in which Cbf1 operates in sulfur amino acid metabolism without being bound to the CDE1 sequences found upstream of the methionine biosynthetic genes [12,13]. These authors mainly justify such a proposal by their Northern analyses that, in contradiction with our original report, fail to reveal any difference in the transcriptional level of two methionine biosynthetic genes, *MET16* and *MET25*, between the cells expressing and those lacking a functional Cbf1 [13]. Because this model contradicts almost all the results described above and still fails to explain the methionine auxotrophy of a *cbf1* disrupted strain, we decided to produce a complete analysis of the transcription of the methionine biosynthetic genes in cells lacking Cbf1 using strictly controlled growth conditions. The results of these experiments are reported in this paper.

2. Material and methods

Strains and Media; The *Saccharomyces cerevisiae* strains used for this work were the *cbf1* disrupted strain CC718–1A (Mat a, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *cbf1*::*TRP1*) and its parental strain W303–1A (Mat a, *ade2*, *his3*, *leu2*, *trp1*, *ura3*). Yeast cells were grown in B medium which was a synthetic medium completely devoid of the sulfur element. Its composition was tas follows: (i) mineral salts: 15 mM ammonium chloride, 6.6 mM monopotassium phosphate, 0.5 mM dipotassium phosphate, 1.7 mM sodium chloride, 0.7 mM calcium chloride and 2 mM magnesium chloride; (ii) oligo-elements: 0.5 μg/ml boric acid, 0.04 μg/ml copper chloride (1 H_2O), 0.1 μg/ml potassium iodide, 0.19 μg/ml zinc chloride and 0.05 μg/ml ferric chloride (6 H_2O); (iii) vitamins and growth factors: 2 μg/ml calcium panthothenate, 2 μg/ml thiamine, 2 μg/ml pyridoxine, 0.02 μg/ml biotin and 20 μg/ml inositol. This medium was filter sterilized and glucose was added to a 2% final concentration. For northern blot analyses, cells were first grown in B medium in presence of a repressing amount (1 mM) of L-methionine as sulfur source.

Northern (RNA) blot analyses. Northern blotting was performed as

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described by Thomas [14] with total cellular RNA extracted from yeast as described by Hoffman and Winston [15].

3. Results

The gene–enzyme relationships in sulfur metabolism are now almost completely deciphered and the corresponding molecular data are available. The biosynthesis of sulfur amino acids first requires the reductive assimilation of sulfate ions, a biochemical process performed through five enzymatic steps (Fig. 1). Since the sulfate ion is fairly stable and cannot be reduced without being activated, the two first commited steps lead to the synthesis of phosphosulfate compounds by means of ATP. These two steps are catalyzed by the products of gene *MET3*and *MET14*. The subsequent reactions are really reductive and convert the sulfur atom into its most reduced form, sulfide. These steps are catalysed by the product of the gene *MET16* and by the products of the genes *MET5* and *MET10* (Fig. 1). The product of the gene *MET25* catalyses the last assimilative step that converts sulfide into organic sulfur in the form of homocysteine, the precursor of both cysteine and methionine.

In *Saccharomyces cerevisiae*, the sulfate assimilation pathway is subjected to a negative regulation: in response to an increase of intracellular *S*-Adenosylmethionine (AdoMet), transcription of methionine biosynthetic genes is turned off. Three transcriptional factors are known to be involved in this regulation: the products of genes *MET4*, *MET28* and *MET30* [10,16,17]. A phenotypic study of cells lacking Cbf1 revealed that such mutants grow when an exogenous supply of organic sulfur is provided but are unable to grow in presence of inorganic sulfur sources [10]. This indicates that the mutation of *cbf1* first impairs the sulfate assimilation pathway. Accordingly, as depicted in Fig. 2, all the structural genes of the sulfate assimilation pathway contain one or two copies of the Cbf1 DNA target sequence in their 5′ upstream regions.

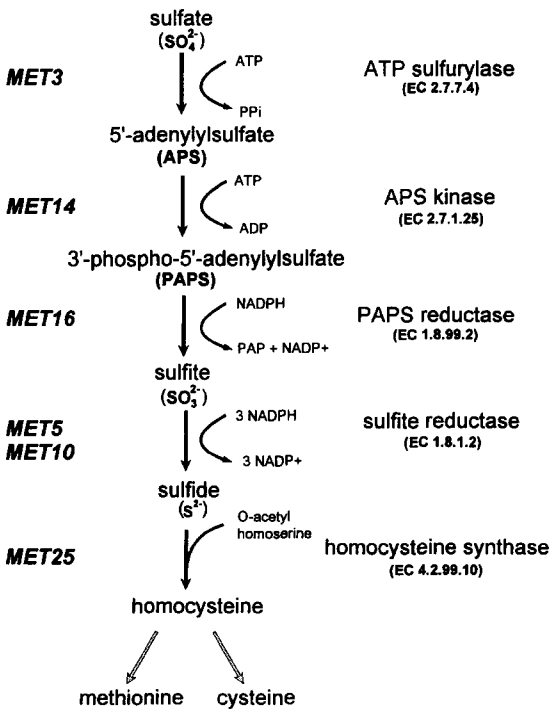

Fig. 1. The sulfate assimilation pathway of *Saccharomyces cerevisiae*.

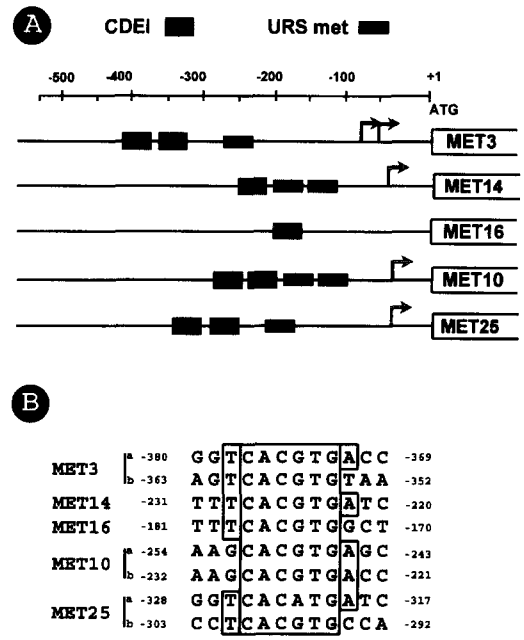

Fig. 2. (A) Organisation of the promoter regions of the methionine biosynthetic genes. The grey and the black boxes represent the CDE1 elements and the repressing element (URS) respectively. The transcription starts are indicated by arrows. (B) Sequences of the CDE1 elements and their adjacent bases.

To determine whether Cbf1 is indeed involved in transcriptional activation of the sulfur network, we and others performed classical steady state Northern experiments by monitoring the level of transcription of two sulfur genes (*MET16* and *MET25*) in a *cbf1* disrupted strain and its parental wild type strain. As reported above, these experiments led to conflicting results [10,12]. We envisaged that these discrepancies could be accounted for by the fact that, in yeast, the intracellular pool of AdoMet is known to exhibit slight variations along the growth curve, increasing at the middle of the exponential growth phase [18]. Because AdoMet is the physiological effector of the transcriptional regulation of the methionine biosynthetic genes, such variations should be of importance in Northern experiments if the growth stage of the differents cultures were not carefully monitored. Furthermore it must be stressed that wild type cells and those lacking *cbf1* differ by their capacities to concentrate the sulfate ions which are present in a large excess in the medium used for these experiments and which are the metabolic precursors of AdoMet. Indeed, *cbf1* mutants lacks sulfate permease activity [10].

We thus devised an experimental growth procedure that, using a specific growth medium with no sulfur element (B medium), suppressed the growth dependent variations of intracellular AdoMet. The two strains (*CBF1* and *cbf1* :: *TRP1*) were first grown in B medium in presence of a repressing amount of L-methionine (1 mM). When the cells reached a density of about 10^7 cells/ml, they were harvested by filtration, washed and then transfered into B medium without methionine. Total RNAs

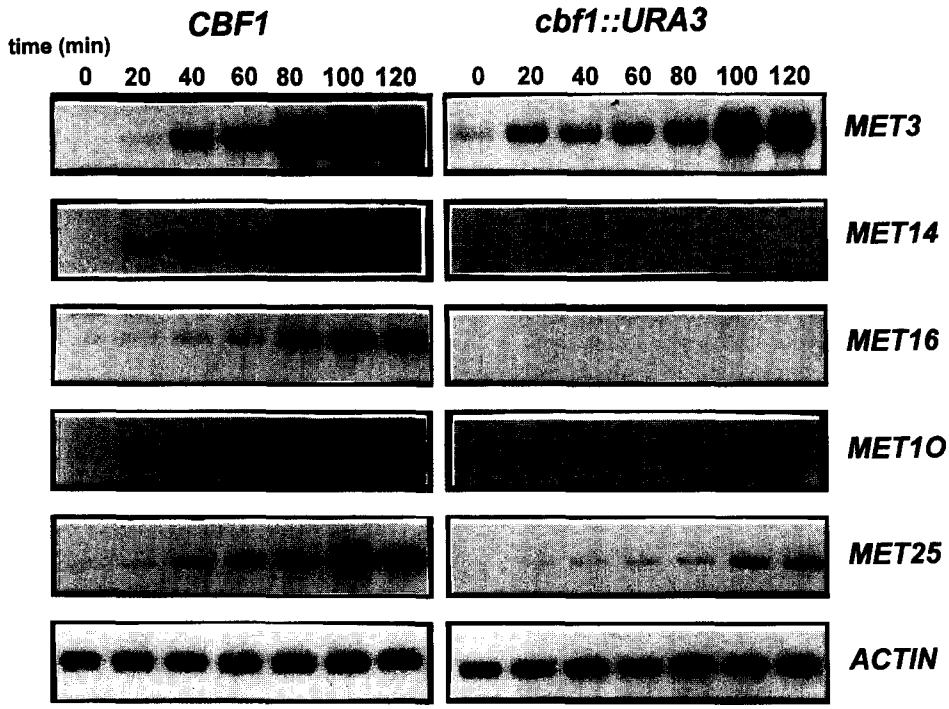

Fig. 3. Derepression kinetics of the transcription of genes *MET3, MET10, MET14, MET16, and MET25* were monitored in wild type cells and in cells lacking Cbf1. The cells used were the strain CC718–1A (*cbf1*::*TRP1*) and its parental strain W303–1A. They were grown in 150 ml of B medium in the presence of a repressing amount (1 mM) of L-methionine as sulfur source. When the cells reached a density of about 10^7 per ml, they were harvested by filtration and washed with 150 ml of B medium. The cells were then suspended in 100 ml of B medium without methionine and shaken at 28°C. Samples were then withdrawn at different times and total RNAs were extracted. For each time, 10 μg of total RNA was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. The transferred RNAs were hybridized to the following radioactive fragments: the *Xho*I–*Bgl*II fragment from the *MET3* gene [22], the *Bgl*II–*Bgl*II fragment from the *MET10* gene [23], the *Hpa*I–*Sca*I fragment from the *MET14* gene [9], the *Cla*I–*Eco*RI fragment from the *MET16* gene [24] and the *Xba*I–*Eco*RI fragment from the *MET25* gene [25]. The actin probe was used as control of the amounts of RNA loaded. The films were exposed for three days at −80°C.

were extracted from these two strains at different time intervals after the shift and the transcriptional derepression of sulfur genes was monitored using probes specific to the *MET3*, *MET14*, *MET16*, *MET10* and *MET25* genes. A probe specific to the actin encoding gene was used as a control of the amount of RNA loaded. In this experiment, the fact that the wild type strain possesses an active sulfate permease, unlike the *cbf1* disrupted strain, is of no consequence to the regulatory state of the sulfate assimilation pathway since the B medium does not contain any sulfate ions.

The results of such an experiment are shown in Fig. 3. This experiment was performed twice yielding the same results. In the wild type strain, the kinetics of transcriptional derepression appear to be the same for all the methionine biosynthetic genes: the mRNAs were first evidenced 20 min after the shift and the transcription reached its maximal level 80 min after the shift. In contrast, in the *cbf1* disrupted strain, no transcription at all was observed for the *MET14* and the *MET16* genes. In addition, in the *cbf1* mutant, only a very low level of transcription of the *MET10* gene was measured while the transcriptional levels of gene *MET3* and *MET25* appears to be 50% lower than those measured in the wild type strain. It must be noted that, as published earlier, the level of ATP sulfurylase (encoded by *MET3*), PAPS reductase (encoded by the gene *MET16*), sulfite reductase (encoded by the gene *MET10*) and homocysteine synthase (encoded by the gene *MET25*) measured in a *cbf1* disrupted strain correlate well with the above reported experiment [10]. All these results prove that Cbf1 is actually involved in the transcriptional activation of the sulfur metabolism.

4. Discussion

This work allows us to clearly establish the physiological causes of the methionine requirement exhibited by yeast cells lacking Cbf1. This work therefore provides reliable bases to understand how Cbf1 functions. Indeed, using a strictly defined protocol for analysing the transcription level of the genes governing the sulfate assimilation pathway, we were able to definitively prove that the expression of at least three of these genes absolutely depends on the integrity of Cbf1. These three genes are *MET10*, *MET14* and *MET16*. In addition, we show that full expression of the gene *MET25* also depends on Cbf1. These results, demonstrating the *trans* effects of *cbf1* mutations, are in accord with those obtained with *cis* CDE1 mutations when they are present upstream of gene *MET14* [9], *MET16* [19] and *MET25* [10]. Moreover these results allow to understand the results of the domain swap experiments performed by Dang and collaborators with chimeric Cbf1-USF fusion protein [20]. Using a functional assay to rescue a *cbf1* disrupted strain from methionine auxotrophy, Dang and collaborators have indeed shown that the basic region of Cbf1 could be replaced by the basic region of either the USF factor or c-*myc*, both of which recognize the sequence CACGTG. In contrast, the methionine auxotrophy of a *cbf1* disrupted strain was not rescued by the

expression of a protein in which the basic region of Cbf1 was replaced by that of factor AP4, which recognizes the sequence CAGCTG. However, selection of methionine prototroph revertants showed that they all expressed a Cbf1–AP4 fusion protein containing a single substitution in the AP4 basic region that allowed it to recognize the CACGTG motif. This also provides a rationale for understanding how the methionine requirement of a *cbf1* disrupted strain could be suppressed by the over-expression of the Pho4 protein in a Pho80 dependent manner [11]. Indeed, Pho4, as Cbf1, belongs to the familly of bHLH DNA binding factors recognizing the core sequence CACGTG. However, it seems that in a wild type strain, flanking bases serve to Pho4 and Cbf1 to discriminate between their respective targets; in particular, most of the CACGTG motifs present upstream of the sulfur genes or centromeres are preceded by a 5′ T while none of those recognized by Pho4 are [21].

All these results prove that specific binding of Cbf1 to the CDE1 sites is essential in carrrying out both its kinetochore-related function and its transcription function. The results presented here fully support this view. However, while necessary, cbf1 binding to the CDE1 sites is not sufficient for inducing the transcriptional activation of the sulfur genes. Accordingly, Cbf1 was shown to lack transcription activation capabilities [10]. We thus favor the hypothesis that CDE1 bound Cbf1 modifies the conformation of chromatin, thus facilitating the assembly of either active transcription complexes or functional kinetochores. This view is supported by the recent results of O'Connell et al. [19] demonstrating that Cbf1 aids the selective removal of nucleosomes from the transcription initiation region of the *MET16* gene.

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