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Evidence for co-regulation of Cu,Zn superoxide dismutase and metallothionein gene expression in yeast through transcriptional control by copper via the ACE 1 factor

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Saccharomyces cerevisiae mutant strain DTY26, lacking ACE1, the protein mediator for the induction of metallothionein gene expression, is unable to increase Cu,Zn superoxide dismutase mRNA in response to copper. In the wild-type strain DTY22 transcription of both Cu,Zn superoxide dismutase and metallothionein genes is induced by copper and silver, as expected on the basis of previous results indicating that ACE1 binds only Ag(I) besides Cu(I). We conclude that at the transcriptional level Cu,ZnSOD is co-regulated with metallothionein. Furthermore, structural similarities between the two promoters were found, which could explain the co-regulation effect and the quantitative differences in the response of the two genes to copper

Cu,Zn superoxide dismutase, Metallothionein, ACE1, Transcriptional regulation, Metal transport, Yeast UAS

1. INTRODUCTION

Superoxide dismutases (SODs) are metalloproteins that catalyze the reaction:

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$$

in a very efficient way [1]. Three SOD isoenzymes have been discovered, containing either Cu(+Zn), or Mn or Fe, as the metal ion that confers catalytic activity to each protein. The three-dimensional structure and the mechanism of action of these enzymes are well known; however, their biological properties are less clear. The Mn-containing form is typical of highly aerobic systems (aerobic bacteria or mitochondria) and is able to change its level in the cell depending on the exposure to varying concentrations of oxygen or to redox active molecules in the presence of oxygen. On the other hand, Cu,ZnSOD activity has been found to increase in yeast in response to oxygen and copper [2,3], although to a lower extent if compared to the effect of oxygen on the Mn-enzyme of yeast mitochondria. We have found [4] that this copper effect is independent of the presence of oxygen, suggesting that the metal affects Cu,ZnSOD gene expression in a more direct way than via previous production of O_2^- by Cu(I)/Cu(II) redox cycling in air. These findings prompted us to investigate in more detail whether the expression of Cu,ZnSOD gene in yeast is regulated by some trans-acting factor which is specifically sensitive to copper ions. Such a system has been described in yeast for metallothionein (MT) gene expression. The constitutively expressed regulatory protein ACE1 specifically binds to the MT gene control sequence in the presence, but not in the absence, of copper. It has been proposed that the induction of MT gene expression in response to increased copper levels in the cell is mediated by a change in ACE1 conformation upon Cu(I) binding, which in turn would increase the affinity of ACE1 for MT gene promoter and cause a many-fold induction of MT gene transcription. Ag(I), but not other metal ions, can also induce MT gene expression via the above mentioned mechanism [5–7].

The results presented in this paper show that the Cu,ZnSOD gene is co-regulated with the MT gene in yeast at the transcriptional level. We propose that this effect is related to the presence in the yeast Cu,ZnSOD promoter of regions similar in nucleotide sequence and positioning to the regions of the MT promoter interacting with ACE1.

2. MATERIALS AND METHODS

2.1 *Chemicals*

T4 polinucleotide kinase and DNA polimerase I, Klenow fragment, were obtained by Boehringer, Mannheim, yeast extract was obtained from Difco; silver nitrate was obtained from Aldrich. All other materials were of reagent grade and were obtained from the best commercial sources available

2.2 *Yeast strains and culture conditions*

Saccharomyces cerevisiae wild-type strain DTY22 and deltaACE1

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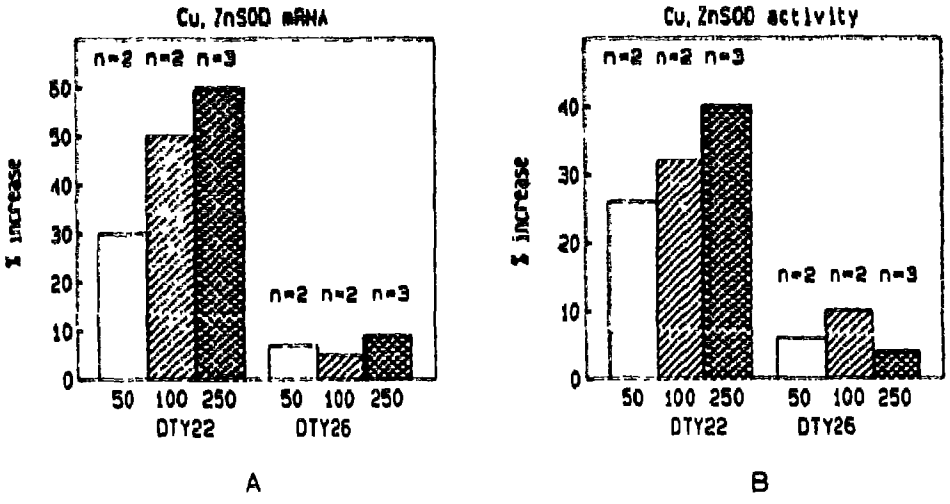

Fig 1 Effect of copper on the levels of (A) Cu,ZnSOD mRNA and (B) Cu,ZnSOD activity in wild-type strain DTY22 and in delta ACE1 mutant strain DTY26 Percent increase upon copper addition are reported with respect to the control (4 μM $CuSO_4$) Copper concentration (μM) and the strains employed are indicated on the abscissa. The number of experiments is also indicated

mutant strain DTY26 were a generous gift of Dr Dennis Thiele and are described in detail elsewhere [8]

Yeast was grown in standard YPD medium (2% glucose, 1% yeast extract, 2% peptone). The concentration of copper in YPD was 4 μM, except when $CuSO_4$ was added to the final concentration indicated in the figures (50, 100 or 250 μM), copper concentrations higher than 250 μM inhibited growth of the mutant strain. Ag(I) was supplied (where indicated) as $AgNO_3$ to reach a final concentration of 50 μM

Cultures were grown from an overnight inoculum in a rotatory shaker kept at 30°C, until they reached late exponential phase

2.3. *Northern blot analysis*

Total RNA was prepared according to the method of Schultz [9] and subject to standard Northern blot analysis on nitrocellulose filters [10] after 1.3% formaldehyde agarose gel electrophoresis as previously described [11] Synthetic oligonucleotides were used as probes for Cu,ZnSOD mRNA (+82 to +111 of the coding sequence, see [12]) and for MT mRNA (+40 to +67 of the coding sequence, see [5]) after standard 5' labelling with [γ^{32}P]dATP [10]. Quantitation of autoradiograms was obtained by densitometric scanning with a LKB Ultroscan XL Laser Densitometer coupled with a LKB 2400 GelScan XL software package Normalization of specific Cu,ZnSOD and MT mRNAs was obtained by evaluation of both major ribosomal RNAs or total RNA content in each sample Ribosomal protein L2 mRNA was evaluated using a 1.2 kb *BglII/XhoI* insert from plasmid pL2A as a probe [13] labelled by the random primer technique [10]

2 4 *Activity assays*

Yeast cell extracts were obtained as follows Cells were washed twice in ice-cold water, suspended in 2 vols of water and mechanically disrupted by repeated vortexing of the suspension with 0 45 μm glass beads The lysate was removed and the glass beads were washed 3 times with 2 vols of water. The resulting supernatant was pooled with the lysate and centrifuged at 35 000 × *g* for 30 min Cu,ZnSOD activity was assayed on the supernatant by a polarographic method [14] at pH 9.9, where MnSOD activity is undetectable, and expressed as μg enzyme/mg total protein with reference to a sample of purified yeast Cu,ZnSOD Protein was determined by the method of Lowry et al [15]

Glutathione peroxidase activity was assayed as previously described [4]

2 5 *Sequence analysis*

Comparison of 5' untranslated regions of Cu,ZnSOD and MT genes was made using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package [16].

3. RESULTS AND DISCUSSION

3 1. *Effect of copper on MT and Cu,ZnSOD gene expression in an ACE1-lacking yeast strain*

Fig. 1 shows that addition of copper to cultures of wild-type strain DTY22 induces an increase in both mRNA transcription and activity of Cu,ZnSOD in a parallel fashion. The level of induction of MT gene transcription is higher (Fig. 2) but follows the same trend. On the other hand, neither activity nor mRNA level of Cu,ZnSOD were increased by copper in the mutant strain lacking ACE1 (DTY26), in which no induction by copper of MT gene transcription was observed

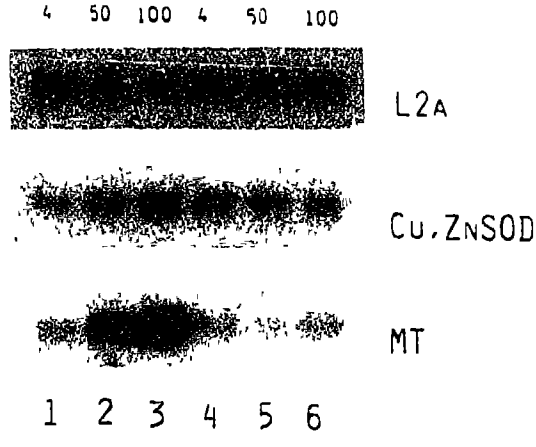

Fig. 2 Northern blot analysis of Cu,ZnSOD, MT and ribosomal protein L2A mRNAs Total RNA was extracted from strain DTY22 (lanes 1-3) and DTY26 (delta ACE1) (lanes 4-6) Copper concentration in the culture medium is indicated above each lane (μM)

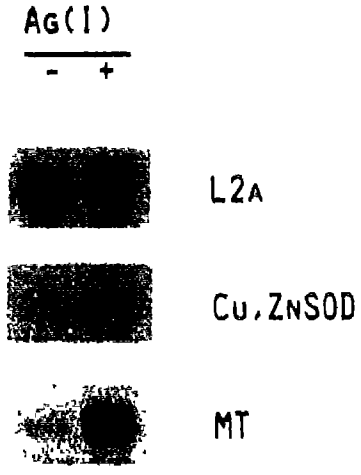

Fig. 3. Effect of 50 μM silver nitrate on the levels of Cu,ZnSOD, MT and L2A mRNAs in wild type strain DTY22.

(Figs. 1 and 2). Glutathione peroxidase activity was previously reported to increase in copper-overloaded yeast cultures [4]. Its activity increased upon copper addition from 2.32×10^{-3} to 6.48×10^{-3} U/mg protein in DTY22 and from 2.31×10^{-3} to 4.38×10^{-3} U/mg of protein in DTY26. Therefore we conclude that the effect produced by the absence of ACE1 seems to be specific for MT and Cu,ZnSOD.

Ribosomal protein L2A mRNA level was independent of copper both in the wild-type and in the mutant, an additional indication of the specificity of copper effect in DTY22 and DTY26. We interpret these results as a strong evidence for the co-regulation of Cu,ZnSOD and MT by ACE1 in yeast.

3.2. *Effect of Ag(I) on MT and Cu,ZnSOD mRNA transcription in wild-type yeast*

A further indication of a common regulatory mechanism at the transcriptional level of both proteins emerges from the experiments performed by growing yeast cells in the presence of silver. The ACE1-mediated regulation of MT in yeast was reported to be specific only for Cu(I) and Ag(I), since no other metal ion stimulate ACE1 binding to MT promoter [6].

Fig. 3 shows that in wild-type yeast, Ag(I) salts cause an induction of Cu,ZnSOD mRNA transcription which again parallels the increase in MT mRNA, while ribosomal protein L2A mRNA is unaffected. Because of the specificity of the silver effect, this is additional evidence that transcription of Cu,ZnSOD gene in yeast is specifically under control of the same system (ACE1) which is responsible for MT regulation.

3.3. *Comparison of MT and Cu,ZnSOD promoter regions in yeast*

We have compared the untranslated 5′ regions of Cu,ZnSOD and MT genes [3,12] by computer-aided sequence analysis. This comparison (Fig. 4) has shown several noteworthy sequence similarities in the regions located upstream the ATG start codon. In particular, two short regions (Fig. 4A) in the 5′ end of the Cu,ZnSOD gene are partially homologous to the upstream activating sequences (UASd and UASc) of the MT promoter, which have been implicated in the bin-

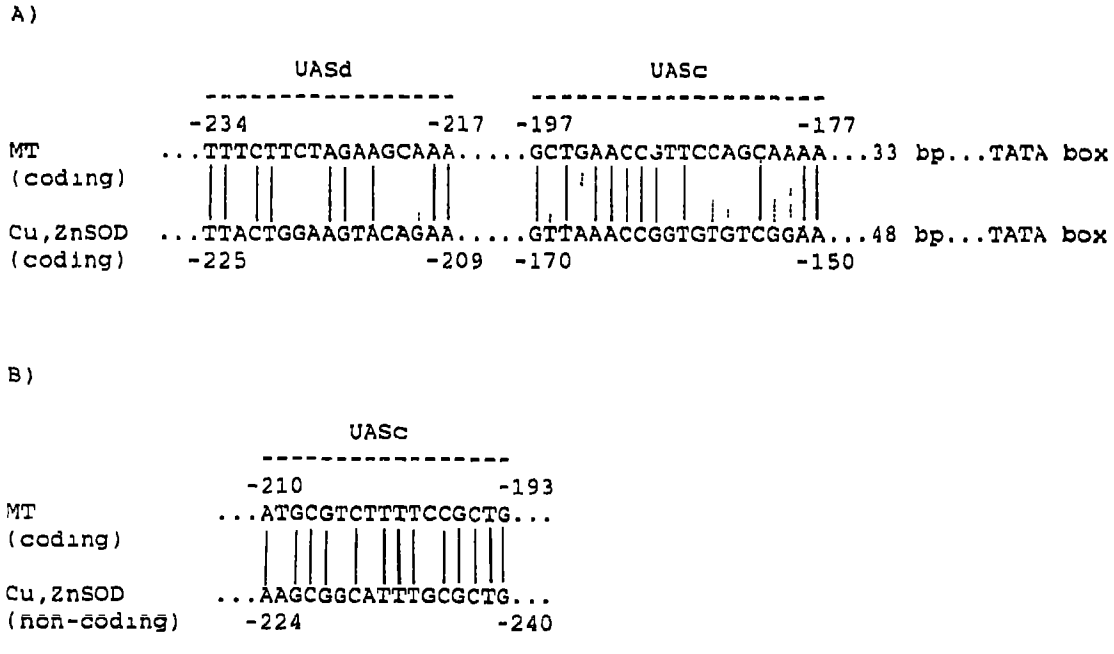

Fig. 4. Sequence similarities between Cu,ZnSOD promoter region and MT UAS regions (ACE1 binding site). Numbering is respect to the ATG start codon. A vertical line indicates coincidence of bases between the two promoters, a dotted line indicates a Pu-Pu or a Py-Py homology.

ding of ACE1 to this promoter [6]. The imperfect UAS sequences of the SOD promoter could cause a lower affinity of ACE1 to this region, resulting in the observed lower induction in the case of SOD mRNA transcription compared to MT mRNA. Furthermore, MT promoter has two (or more) potential ACE1 binding-sites (not shown), while SOD has only one.

Noticeably, an almost perfect UAS sequence (Fig. 4B) is present on the non-coding strand of Cu,ZnSOD promoter, that is in the opposite orientation with respect to the ATG start codon. This fact could even impair ACE1-mediated induction by inappropriate binding of the protein on the incorrect DNA strand. Indeed, it has been reported that the orientation of other UAS sequences in yeast is crucial for the level of activation of transcription [17]. The unavailability of yeast Cu,ZnSOD gene to our laboratory did not allow us to complement the in vivo evidence obtained from the results described above with footprint, band shift and methylation interference assays. Such in vitro controls have been performed by another group, and confirm that ACE1 can bind the SOD promoter efficiently (J. Valentine and D. Thiele, personal communication).

A further comment is deserved by the different level of induction of Cu,ZnSOD compared to MT. The above discussed differences in promoter sequences could per se explain a less efficient binding of ACE1 to Cu,ZnSOD gene and a consequent lower induction of this gene.

The physiological role of the co-regulation of SOD and MT in cells exposed to excess copper remains to be elucidated, but the results reported here point to a metal-buffering role of Cu,ZnSOD as a part of its antioxidant activity.

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