MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 1158–1163
0270-7306/86/041158-06$02.00/0
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Vol. 6, No. 4

Tandemly Duplicated Upstream Control Sequences Mediate Copper-Induced Transcription of the *Saccharomyces cerevisiae* Copper-Metallothionein Gene

DENNIS J. THIELE AND DEAN H. HAMER*
Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20892

Received 22 October 1985/Accepted 16 January 1986

Transcription of the *Saccharomyces cerevisiae* copper-metallothionein gene, *CUP1*, is inducible by copper. By analyzing deletion and fusion mutants in the *CUP1* 5'-flanking region, we identified two closely related, tandemly arranged copper regulatory elements. A synthetic version of one of these elements conferred efficient copper induction on a heterologous promoter when present in two tandem copies.

The *CUP1* locus is an important determinant of copper resistance in *Saccharomyces cerevisiae*. The purified protein product of the *CUP1* gene (25) is 53 amino acids long, contains eight copper ions ligated to 12 cysteine residues, and lacks an eight-amino-acid amino-terminal hydrophobic region predicted from the DNA sequence (1, 4, 14). The purified protein product can also bind in vitro to eight atoms of silver or to four atoms of zinc or cadmium, although it is not known to play any role in the metabolism of these ions in vivo (25). Although the *CUP1* gene product bears only limited primary sequence homology to the mammalian metallothioneins (MTs), the two types of protein are similar in their small size and unusual composition. Moreover, recent gene replacement experiments have shown that either of two monkey MT isoforms can restore copper resistance to *S. cerevisiae* that lacks the endogenous *CUP1* gene (D. J. Thiele, N. J. Walling, and D. H. Hamer, Science, in press). For these reasons, we refer to the *CUP1* gene product as "copper-MT," rather than the previously used term "copper-chelatin."

The expression of *CUP1* is controlled by two distinct mechanisms, gene amplification and transcriptional induction. Most laboratory strains of *S. cerevisiae* are stably copper resistant (*CUP1*^{R}) and contain 10 to 20 copies of *CUP1* on a tandemly amplified 2-kilobase reiteration unit. Other strains are copper sensitive (*cup1*^{s}) and contain only a single copy of the *CUP1*-coding sequences (4). The levels of copper-MT are directly related to gene copy number in such strains (24).

Copper-MT levels are also elevated in strains that have been briefly exposed to high levels of copper ions in the media, and this elevation is accompanied by a rapid rise in the amount of *CUP1* mRNA (14, 24). Studies with fusion genes have shown that this rise is due to induction at the transcriptional level and that 430 base pairs (bp) of 5'-noncoding DNA is sufficient to generate the response (1, 10). Transcription from these upstream regulatory sequences is also affected by the status of the endogenous *CUP1* locus. Strains lacking *CUP1*-coding sequences constitutively transcribe an episomal *CUP1* fusion gene (10).

The mechanism by which copper induces *CUP1* gene transcription and the precise role of copper-MT in this process are unknown. In the present work, we constructed deletion mutants and synthetic hybrid promoters to localize *cis*-dominant control elements that may serve as interaction sites for copper-dependent transcriptional factors. Our results show that two closely related, tandemly arranged upstream sequences play a critical role in the regulation of *CUP1* gene expression by copper.

MATERIALS AND METHODS

Cells and growth conditions. *S. cerevisiae* BR10 (*MATa gal1 trp1-1 his4 CUP1*^{R} Ade$^-$) was used in all studies. This strain was grown in synthetic glucose medium, appropriately supplemented, at 30°C.

Construction of deletions in *CUP1* 5'-flanking sequences. The starting plasmid, RC4, contains a *CUP1-galK* fusion gene carried on an *Escherichia coli-S. cerevisiae* vector which contains the *S. cerevisiae TRP1* gene as a selectable marker, with *ARS1* and *CEN3* sequences to promote stable, low-copy-number replication (10). Deletions extending in from the 5' direction were obtained by partial digestion of RC4 with *Xba*I, resection with exonuclease BAL 31, *Bam*HI linker ligation, *Bam*HI cleavage, and circularization. Digestions extending in from the 3' end of the *CUP1* fragment were contructed by digestion of RC4 with *Eco*RI, resection with exonuclease BAL 31, and end repair with the Klenow fragment of DNA polymerase I. After *Bgl*II digestion and agarose gel purification of the *Bgl*II-blunt-ended fragment, this fragment was ligated to a gel-purified *Bgl*II-*Sma*I fragment from plasmid BCRCΔ4. Plasmid BCRCΔ4 is a derivative of plasmid YCpR1 (18). BCRCΔ4 was constructed by cleaving YCpR1 with *Xho*I, flush ending the termini with the Klenow fragment of DNA polymerase I, and ligating *Bam*HI linkers. After cleavage with *Bam*HI, the plasmid was ligated under conditons that favor circularization, and the ligation mixture was transformed into *E. coli*. The resultant plasmid contained a *CYC1-galK* fusion in which all *CYC1* sequences upstream of −178 were deleted, resulting in the removal of the *CYC1* gene transcriptional regulatory elements (7). The BCRCΔ4 *Bgl*II-SmaI fragment supplied the iso-1 cytochrome *c* (*CYC1*) gene transcriptional initiation sites. Internal deletions were constructed by mobilizing the *Bam*HI fragment from 3' deletion constructs into the *Bam*HI site of 5' deletion constructs. All plasmids were transformed into *E. coli* MC1061 and analyzed by standard restriction enzyme

* Corresponding author.

digestion techniques (15), and the deletion endpoints were determined by dideoxynucleotide (19) or Maxam-Gilbert (16) DNA sequencing techniques.

Construction of synthetic upstream derivatives. Complementary 39-mer oligonucleotides corresponding to the *CUP1* proximal upstream activator sequence (UAS_P) region were synthesized with *Bam*HI sticky ends such that insertion into a *Bam*HI site within a plasmid would generate only a single *Bam*HI site. The following oligonucleotides were synthesized: 5'-GATCCGTCTTTTCCGCTGAACCGTTCCAGCA AAAAAGAC-3' and 3'-GCAGAAAAGGCGACTTGGCA AGGTCGTTTTTTCTGCTAG-5'. This synthetic oligonucleotide duplex DNA was inserted into the *Bam*HI site of BCRCΔ4 at a position 178 residues upstream from the *CYC1* +1 site. Insertion was carried out by the linker tailing method as described previously (22). For double oligonucleotide insertions, the constructs bearing a single insertion in the desired orientation were linearized with *Bam*HI, and a second synthetic upstream activator sequence (UAS) unit was inserted by linker tailing. The number and orientation of oligonucleotide insertions were verified by restriction enzyme analysis and dideoxynucleotide sequencing (19) after subcloning fragments into M13 vectors (17).

Galactokinase assays. Plasmid constructs were introduced into strain BR10 by spheroplast transformation (20). Prototrophs (*TRP*$^+$) were selected and purified for single colonies. Liquid cultures (5 ml) were grown in synthetic glucose medium (to select for plasmid maintenance) to an optical density at 650 nm of approximately 1. Aliquots (1 ml) of each culture were untreated or induced at 30°C with shaking for 30 min in the presence of 0.5 mM $CuSO_4$. Cells were pelleted, suspended in 50 μl of 40% dimethyl sufoxide, and incubated at room temperature with vigorous shaking for 30 min to permeabilize cells. A 5-μl portion of each sample was assayed for galactokinase activity as described previously (10). Incubations were carried out at 37°C for 20 min.

RNA analyses. Cultures (400 ml) for RNA analyses were grown and induced as described for galactokinase assays. Cells were harvested at 4°C and washed with 25 ml of ice-cold sterile H_2O, and total RNA was isolated as described previously (11). S1 nuclease mapping was performed as described previously (23) with 25 μg of total RNA per sample. The double-stranded DNA probe was a 270-bp fragment from BCRCΔ4 which extended from the *Bst*NI site in the *CYC1* 5'-nontranscribed region to the 5'-end-labeled *Nar*I site within the *E. coli* galactokinase structural gene. Samples were fractionated on a 6% polyacrylamide-urea gel, and the dried gel was exposed to X-ray film at −70°C with an intensifying screen.

RESULTS

Identification of a copper-responsive region by deletions in *CUP1* 5'-flanking DNA. A 430-bp fragment of *CUP1* 5'-flanking, untranslated DNA was previously shown to contain all of the information required for transcriptional initiation and copper induction (1, 10). We constructed plasmids which contain specific deletion mutations within this region to delineate and identify sequences involved in transcriptional induction. The *CUP1* promoter mutants were fused to coding sequences of the *E. coli* galactokinase gene (*galK*), thus facilitating analyses of the effects of these mutations on expression levels and regulation. The deletion constructs were introduced into a *CUP1*^{R} *gal1* strain to ensure that cells would be viable upon induction with copper and that any

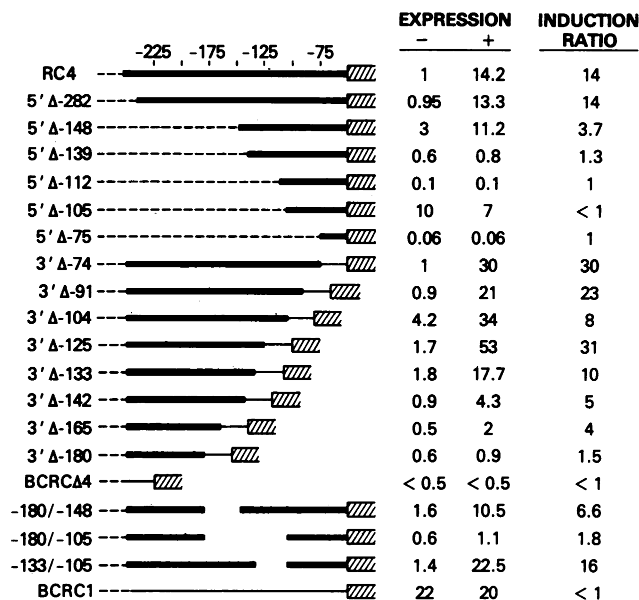

FIG. 1. Mutational analysis of *CUP1* upstream sequences. Deletions extending from 5' and 3' directions or internal deletions were constructed and assayed for galactokinase activity as described in Materials and Methods. All values were standardized to the basal level of expression of plasmid RC4 (assigned a value of 1). Each construct was assayed at least three times in two or more independent transformants. Plasmid BCRC1 is a *CYC1-galK* fusion (18) which contains *CYC1* 5'-flanking sequences out to −2,000 and therefore harbors intact *CYC1* UAS. (8). Plasmid BCRCΔ4 contains *CYC1* 5'-flanking sequences only to position −178 and therefore lacks the UASs.

detectable galactokinase activity would be solely derived from the *E. coli galK* enzyme.

The effects of deleting specific *CUP1* upstream sequences on expression and regulation are shown in Fig. 1. In all experiments, basal-level expression values were expressed relative to the uninduced level of expression of the starting plasmid, RC4, which was arbitrarily assigned a value of 1. Deletions which progressed in from the 5' direction demonstrated that wild-type levels of expression and regulation were maintained up to a position 282 bp from the *CUP1* cap site (mutant 5'Δ-282). Nearly wild-type induced levels were obtained with mutant 5'Δ-148, although basal-level expression increased approximately threefold. A deletion extending to −139 resulted in a very low level of induction, but beyond this position no induction was observed for any of the mutants. An unexpected result, however, was the extremely high basal level of expression in mutant 5'Δ-105, which had an uninduced galactokinase activity 10-fold higher than that of the starting plasmid, RC4. This high basal level was lost in the next mutant, 5'Δ-75, which was transcribed 20-fold less efficiently than the intact gene. The low expression in this mutant may be attributable to the deletion of TATA box sequences at position −77.

The scheme utilized to construct deletions extending in from the 3' end of the *CUP1* upstream region resulted in the loss of the *CUP1* TATA sequences and cap sites. We therefore substituted the *CYC1* TATA region and mRNA start sites for the corresponding region of *CUP1* in the 3' deletions. The truncated *CYC1* promoter used for these experiments lacks its upstream activation elements (8) and was expressed at a low level in both the absence and the presence of copper (Fig. 1, plasmid BCRCΔ4). The addition

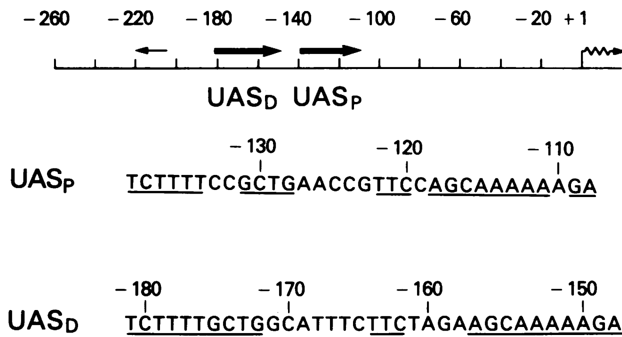

FIG. 2. Schematic diagram of the *CUP1* upstream control region, UAS_P, and UAS_D. The positions of UAS_P and UAS_D are indicated by thick arrows. The presence of a degenerate UAS sequence in an orientation opposite to UAS_P and UAS_D is represented by the thin arrow. The upstream-most start site of transcription is represented by the wavy arrow at +1. UAS_P and UAS_D are shown with homologous sequences underscored.

of *CUP1* sequences from position −74 to position −393 (mutant 3'Δ-74) rendered expression 30-fold inducible by copper. Mutant 3'Δ-91 showed a qualitatively similar response, albeit a lower level of induced activity was obtained. The deletion of sequences to position −104 elicited a high basal level of galactokinase activity, but copper induction was similar to that in mutants 3'Δ-74 and 3'Δ-91. We interpret the results of this deletion to indicate that it retains sequence elements involved in copper induction but is altered at an overlapping or separate region possibly involved in maintaining a low basal level of expression. It is unlikely that the high basal level of expression of this mutant is an artifact of the particular *CUP1-CYC1* junction that is formed, because a similar phenomenon was observed for mutant 5'Δ-105, which forms a completely different junction. Appropriate basal and induced levels were reestablished in mutant 3'Δ-125, but the loss of sequences out to position −133 conferred a lower induced level of expression of the *CYC1* promoter region. Further decreases in induced levels of expression were observed for the 3'Δ constructs 3'Δ-142, 3'Δ-165, and 3'Δ-180, for which 5-, 4-, and 1.5-fold inductions by copper were observed, respectively.

The localization of the copper regulatory sequences was further studied by the construction of internal deletions that retained *CUP1* TATA and start sequences but lacked upstream regions (Fig. 1). A deletion from −105 to −108 showed a very low induction ratio corresponding to that of the 3'Δ-180 parent. However, substantial copper induction was observed in two smaller deletions within this region, −180 to −148 and −133 to −105. This result suggests either that there are two functional elements within the region from −180 to −105 or that all of the information for copper induction lies between −148 and −133. Inspection of the 5' and 3' deletion data supports the first hypothesis. In particular, note that at least partial copper induction was found in both mutant 5'Δ-148 and mutant 3'Δ-165, despite the fact that these two mutants share no common sequences. This observation strongly suggests the presence of multiple functional regulatory elements.

Activation of the *CYC1* promoter by synthetic control sequences. The above results showed that sequences from positions −180 to −105 are essential for efficient copper induction and that this region contains at least two functional elements. A search of this region for internal homologies revealed the presence of two related, tandemly arranged sequences, one centered at position −123 and the other centered position −163. Figure 2 shows the sequences and

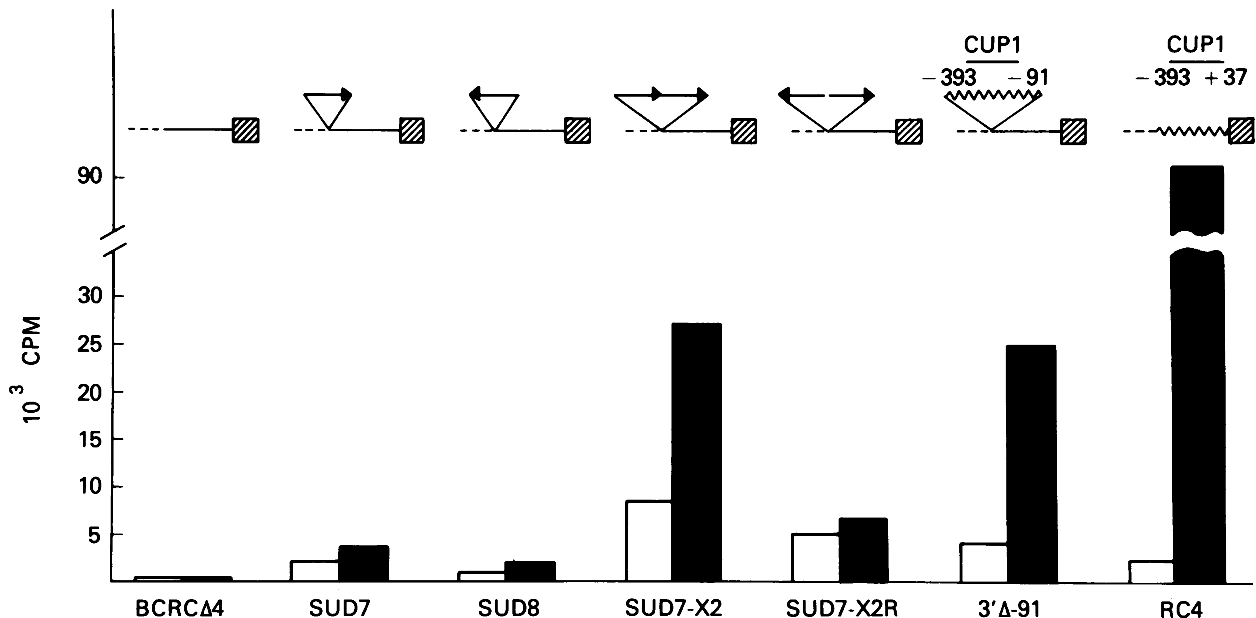

FIG. 3. Copper induction in synthetic upstream derivative (SUD) constructs. Transformants harboring the indicated plasmid construct were purified, grown, and assayed for galactokinase activity as described in Materials and Methods. A schematic representation of each construct is shown above the assay results. Dashed lines represent *CEN3* sequences. Solid lines represent the *CYC1* promoter sequence from −178 to the point of translational fusion to the *E. coli galK* gene (represented by the hatched region). Synthetic insertions and the orientation of each are indicated by arrows. *CUP1* sequences are represented by wavy lines. The counts per minute of [^{14}C]galactose 1-phosphate are indicated on the abscissa.

positions of these elements, which were tentatively designated UAS_P and distal UAS_D. To test the function of these putative regulatory sequences, we synthesized an oligonucleotide copy of UAS_P and inserted it upstream of the truncated *CYC1* promoter fused to *galK*. By using an oligonucleotide in which only one terminus is a *Bam*HI site, we found it possible to construct either single or double insertions. *S. cerevisiae* was transformed with these constructs and assayed for galactokinase activity in the presence or absence of copper. The insertion of a single synthetic UAS_P element in the natural *CUP1* orientation (construct SUD7) resulted in a 10-fold acitvation of basal expression levels and a 1.8-fold induction by copper (Fig. 3). The same element in the reverse orientation (construct SUD8) resulted in a similar 2-fold induction by copper, but absolute basal and induced expression levels were only 50% of those observed in SUD7. Since two UAS elements are present in the authentic *CUP1* regulatory region, we inserted two synthetic UAS_P elements in tandem adjacent to the truncated *CYC1* promoter sequence. This construct (SUD7-X2) resulted in a 40-fold activation of basal transcription and a 3.3-fold induction ratio, as determined by galactokinase activity. When the second upstream element was inserted in the reverse orientation (SUD7-X2R), basal transcription was elevated 24-fold, but copper treatment had little effect. As a control for the efficiency of these synthetic constructs, we used mutant 3'Δ-91, in which all the *CUP1* regulatory sequences from positions −91 to −393 are fused to the same *CYC1* sequences used for the oligonucleotide fusions. This construct resulted in approximately the same induced expression level as the double tandem insert SUD7-X2, suggesting that the synthetic elements are capable of a full response to copper. However, the basal level of expression in mutant 3'Δ-91 was approximately 2-fold lower than that in SUD7-X2, suggesting a possible role of additional sequences in maintaining low transcription levels in the absence of copper. Interestingly, none of the fusions to *CYC1* resulted in the high level of copper-induced transcription observed for the authentic *CUP1* promoter containing its natural TATA and cap sequences.

The results obtained from the galactokinase assays were confirmed by S1 nuclease mapping of the RNA from cells carrying the synthetic hybrid promoters. These experiments utilized a probe end labeled in the *galK*-coding sequences and extending through *CYC1* promoter region. The transcripts derived from the synthetic constructs had the same complex series of 5' ends as those derived from the authentic, intact *CYC1* promoter (plasmid BCRC1) (Fig. 4). Moreover, the relative levels of these transcripts paralleled the galactokinase levels shown in Fig. 3. Thus, the synthetic *CUP1* regulatory element serves to increase transcription from the normal *CYC1* start sites in a copper-dependent fashion. The S1-nuclease-protected fragments derived from the RC4 transcripts correspond to the *galK*-coding sequences in this construct.

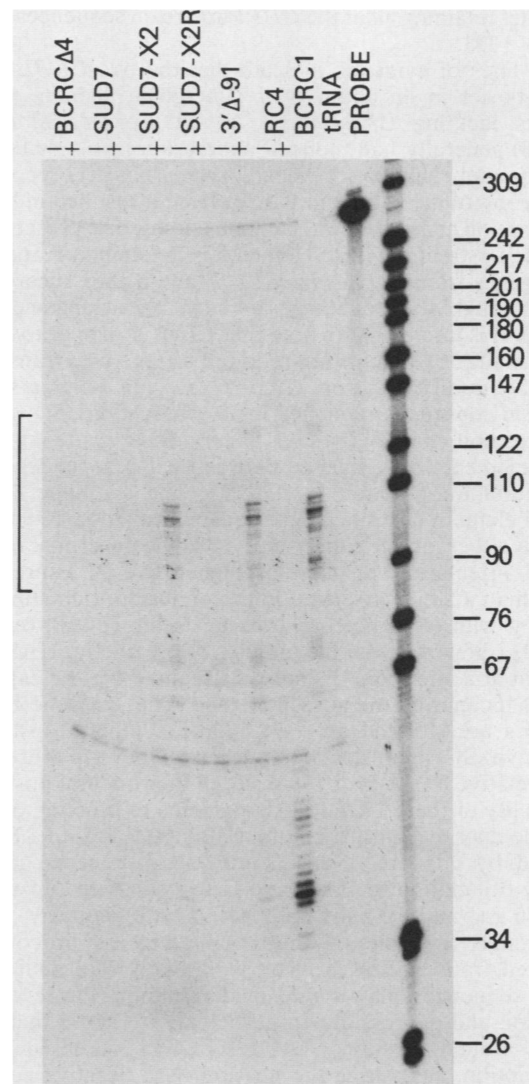

FIG. 4. Copper-inducible transcripts arising from synthetic *CUP1* UAS_P-*CYC1* constructs initiate at the authentic *CYC1* start sites. Start sites for mRNA molecules derived from the indicated constructs (shown in Fig. 3) were located by S1 nuclease analysis. Assays were carried out as described in Materials and Methods with a 270-bp *Bst*NI-*Nar*I fragment which was 5' end labeled at the *Nar*I site, internal to the *E. coli galK* gene. − and + indicate RNA samples prepared from control cells or transformants induced with 0.5 mM $CuSO_4$ for 60 min at 30°C, respectively. BCRC1 indicates RNA prepared from uninduced cells transformed with a *CYC1-galK* fusion in which *CYC1* UASs were retained. tRNA indicates the addition of 25 μg of calf liver tRNA to the sample. The unmarked lane to the far right contains ^{32}P-labeled pBR322-*Msp*I marker fragments. Transcripts derived from the *CYC1* start sites are bracketed; these represent authentic *CYC1* start sites in agreement with those mapped previously (8). Numbers at the right are base pairs.

DISCUSSION

We identified a duplicated upstream DNA sequence involved in the copper-induced transcription of the *S. cerevisiae CUP1* gene. Analysis of 5', 3', and internal deletion mutants established that sequences between −105 and −180 are essential for efficient copper-induced expression and that there are at least two functional elements within this region. A search of the relevant sequence revealed the presence of two related sequences of 32 and 34 nucleotides, denoted UAS_P and UAS_D respectively. Direct evidence for the function of UAS_P was obtained by placing synthetic copies of this element adjacent to a truncated *CYC1* promoter devoid of its natural UASs (7, 8). The presence of two tandem copies of UAS_P activated transcription from the authentic *CYC1* start sites in a copper-responsive fashion. Moreover, the level of copper-induced expression in the synthetic construct was the same as that in a control

construct retaining all of the *CUP1* upstream sequences from −91 to −393.

Two lines of evidence indicate that the two *CUP1* UAS elements act in an additive or synergistic fashion. First. mutants lackiing UAS_P (e.g., Δ3′-133) or UAS_D (e.g., 5′Δ-148) generally had induced expression levels that were approximately half that of the wild-type promoter. Second, a single copy of the synthetic UAS_P element resulted in lower transcription and induction than two copies. The presence of multiple upstream control elements is a common feature of many genes from *S. cerevisiae* (3, 7) and higher eucaryotes (9). Although the advantage to such an arrangement is unclear, it is interesting to note that *CUP1* is similar to *HIS4* in that multiple elements are required for maximal transcription (12) but different from *GAL1-GAL10*, in which a single synthetic consensus sequence is fully functional (5).

A common characteristic of *S. cerevisiae* upstream regulatory elements described to date is their independence of orientation for function (6, 12, 21). The synthetic *CUP1* control element described here does not function equally as well in either orientation, since a single insertion in the natural orientation resulted in higher overall expression levels than a single insertion in the opposite orientation. In common with other *S. cerevisiae* upstream control regions (12, 21), however, is the ability of synthetic UAS_P to function at a site more distant (upstream) from the cap site than its location in the authentic gene. The synthetic UAS_P elements are situated at −178 in the synthetic upstream derivative (SUD) constructions, whereas they are present at −108 relative to the *CUP1* cap site in their natural position. The ability of these *CUP1* UAS elements to function over a distance may explain why mutant 3′Δ-180 is 1.5- to 1.8-fold induced by copper. A degenerate UAS sequence in the reverse orientation of UAS_P and UAS_D is located upstream of −180 and may be marginally activated by copper.

Although the role of the UAS elements in copper-mediated transcription is clear, it is likely that additional *CUP1* sequences play a role in determining the levels of induction and expression. In particular, we noted that mutations around position −104 resulted in increased basal transcription, suggesting the possibility of negative control sequences in this region. We also noted that all of the *CYC1* hybrid promoters had lower induction ratios and maximal expression levels than the authentic *CUP1* promoter, raising the possibility that sequences downstream of position −91 also play some role in regulation.

There are interesting similarities and differences between the control sequences of the *S. cerevisiae* copper-MT gene and of mammalian MT genes. In both cases, heavy-metal induction is mediated by a sequence that is present in multiple copies in the 5′-flanking region (2, 13, 22) and that appears to function most effectively in its natural orientation (B. Felber and D. Hamer, submitted for publication). Another similarity is that whereas a single copy of the control sequence responds to some extent to heavy metals, multiple copies are required for maximal efficiency. On the other hand, a comparison of the primary sequences of the *S. cerevisiae* and mammalian control sequences does not reveal any obvious extended homologies. Thus, although the arrangement of *cis*-acting control sequences is quite similar, the *trans*-acting regulatory factors that interact with them are likely to be quite different.

ACKNOWLEDGMENTS

We thank Mary Jane Walling for excellent technical assistance, M. Brownstein for oligonucleotide synthesis, C. F. Wright and R. Dilauro for critically reading the manuscript, and Nadine Horne and Carolyn Ray for help in the preparation of the manuscript.

D.J.T. was supported by Damon Runyon-Walter Winchell Cancer Fund Fellowship DRG-843.

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