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Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences

(regulation/promoter/transcription/chromosomal footprinting)

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ABSTRACT **Copper-inducible transcription of the yeast metallothionein gene (*CUP1*) occurs by means of cis-acting upstream activation sequences (UAS) and trans-acting cellular factors. We have used a high-resolution chromosomal footprinting technique to detect the interaction of cellular factors with UAS_{CUP1}. Our results demonstrate that one or more cellular factors bind to UAS_{CUP1} in a copper-inducible fashion. This copper-inducible binding is enhanced in a yeast strain that harbors several copies of the positive regulatory gene *ACE1* and is not detectable in yeast cells that contain a nonfunctional (*ace1-Δ1*) locus. The correlation between yeast metallothionein gene activation and copper-inducible occupation of UAS_{CUP1} sequences suggests that the binding of metallothionein transcriptional regulatory factors to cis-acting control sequences is copper-inducible.**

The metallothioneins (MTs) are a family of low molecular weight, cysteine-rich metal binding proteins that are found in a wide range of eukaryotes (1–3). The metallothionein protein of the yeast *Saccharomyces cerevisiae* is encoded by the *CUP1* gene, a dominant locus that confers copper resistance to yeast cells (4). Copper resistance via *CUP1* expression is mediated by two distinct mechanisms, gene amplification and transcription induction (5–8).

The biosynthesis of yeast MT is regulated at the level of transcription induction in response to high environmental copper concentrations (6–8). *CUP1* promoter fusion experiments have demonstrated that all of the cis-acting sequences necessary to promote copper-inducible transcription are located within 390 base pairs (bp) upstream of the transcription start site (7, 8). Furthermore, *CUP1* promoter deletion mutagenesis studies have localized sequences between approximately −220 and −105 that are essential for copper-inducible transcription (8). A synthetic 32-bp portion of this *CUP1* upstream activation sequence (UAS_{CUP1}) was shown to render a heterologous yeast promoter copper-inducible (8).

Recently, a yeast strain bearing a recessive mutation in a trans-acting regulatory gene for *CUP1* transcription has been isolated (9). The *ACE1* gene (activation of *CUP1* expression) is essential for copper-inducible transcription of *CUP1*; however, the precise role the *ACE1* gene product plays in *CUP1* transcription has not been determined. In this report we demonstrate enhanced occupation, by one or more cellular factors, of UAS_{CUP1} in copper-induced wild-type cells. The copper-inducible binding requires the presence of a functional *ACE1* gene.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. The yeast strains DTY22 (*MATα his6 ura3-52 LEU::YipCL* $CUP1^{R-3}$), DTY26 (*MATα his6 LEU2::YipCL ace1-Δ1::URA3* $CUP1^{R-3}$), and H9 (*MATα his6 leu2*$^-$ *ura3-52* $CUP1^{R-3}$) were utilized in this work (9). DTY22 and DTY26 are derived from strain H9 as follows: DTY22 contains a *CUP1–lacZ* fusion YipCL integrated at *leu2*; DTY26 was constructed from DTY22 by replacing a 1-kb *Bgl* II DNA restriction fragment from the chromosomal *ACE1* locus with a 1.2-kb restriction fragment containing the yeast *URA3* gene. The resultant mutant allele is denoted *ace1-Δ1* and renders these cells defective in copper-inducible *CUP1* gene transcription (9). All strains were grown in synthetic complete (SC) medium minus leucine (10) at 30°C, 300 rpm. Copper induction was carried out on logarithmic-phase cultures ($OD_{650} \approx 0.6$–1.2) by the addition of $CuSO_4$ to 100 μM, followed by incubation at 30°C, 300 rpm for 45 min.

Plasmids. The high copy-number plasmid YEp-ACE1 was constructed by subcloning a 1.8-kb *Hin*dIII DNA restriction fragment containing the *ACE1* gene into the *Hin*dIII site of YEp13 (11). This 1.8-kb *Hin*dIII fragment fully complements the *ace1-1* mutation (M. Szczypka and D.J.T., unpublished data). Plasmids were introduced into yeast strains by transformation as described (12).

Analysis of *CUP1* mRNA. Prior to chromosomal footprinting, cells from 1.5 ml of each culture were harvested and washed with 1 ml of ice-cold RNA extraction buffer. Total RNA was isolated from each cell pellet as described (13). Ten micrograms of each RNA preparation was fractionated by electrophoresis on a 1.5% agarose/formaldehyde gel, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled *CUP1/Xba* I/*Kpn* I 700-bp DNA restriction fragment (14). Blots were washed and exposed to Kodak XAR5 x-ray film at −70°C with intensifying screens. RNA levels were quantitated by excising radioactive bands from nitrocellulose and counting in a scintillation counter. Yeast *PYK1* mRNA levels served as controls for the amount and integrity of RNA loaded onto each lane of RNA gels (9).

Chromosomal Footprinting. Chromosomal DNase I footprinting of the *CUP1* promoter region was carried out by modification of the method previously described (15). Cells from 250 ml of uninduced or copper-treated yeast cultures were pelleted and washed for 5 min in 15 ml of 40 mM EDTA/90 mM 2-mercaptoethanol and resuspended in 8 ml of 1 M sorbitol/1 mM EDTA/3 mM dithiothreitol/2 mg of zymolyase 20T per ml (ICN). Cells were incubated at 30°C for 35 min while shaking at 130 rpm. Spheroplasts were harvested, resuspended in 1.2 ml of lysis buffer (100 mM NaCl/6 mM Tris·HCl, pH 7.4/6 mM $MgCl_2$/6 mM 2-mercaptoethanol/0.15% Nonidet P-40), and treated with 10 strokes of a Dounce homogenizer. Samples of cell lysate (0.3 ml) were immediately removed to 1.5-ml Microfuge tubes containing 30 μl of DNase I at several concentrations up to 0.9 mg/ml. DNase I digestions were carried out at room temperature for 5 min and terminated by the addition of 0.33 ml of stop solution (50 mM

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Abbreviations: UAS, upstream activation sequence; MT, metallothionein.

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Tris·HCl, pH 7.4/1 M NaCl/50 mM EDTA/2% sodium dodecyl sulfate). Yeast genomic DNA was purified by phenol/chloroform extraction, treated with RNase A, and precipitated with isopropanol as described (15).

DNase I cleavage patterns of purified genomic DNA were assayed by extension of 5'-end ^{32}P-labeled oligonucleotide primers hybridized to DNA samples as described (15) with modifications. Modified phage T7 DNA polymerase (16) was used in place of avian myeloblastosis virus reverse transcriptase because it results in fewer artifactual terminations. Modified T7 DNA polymerase was prepared as described (16). Also, multiple rounds of primer-extension were performed and 10–15 μg of genomic DNA was used per reaction to increase the amount of radiolabeled extension products. Primer-extension reactions were performed as follows: genomic DNA (10–15 μg) was combined with 3 μl of 10× reaction buffer (0.2 M Hepes, pH 7.5/0.1 M $MgCl_2$/0.5 M NaCl), 3 μl of 10× dNTP mix (2.5 mM dATP/dCTP/dGTP/dTTP), 1.5 μl of 0.1 M dithiothreitol, ≈50,000 dpm of 5'-end-labeled (^{32}P) oligonucleotide primer, and water to 28 μl. The mixture was heated at 95–100°C for 5 min and then placed at 45°C for 5 min; 1 μl (10 units) of modified T7 DNA polymerase was added. Reactions were incubated at 45°C for 4 min. Additional rounds of primer-extension (usually three) were performed by heating the reaction mixture to 95–100°C for 5 min and hybridizing and extending as described above. Primer-extension reactions were terminated by the addition of 4 μl of a solution containing 0.1 M EDTA, 1% sodium dodecyl sulfate, and 1 mg of proteinase K per ml. Samples were then precipitated with ethanol and fractionated on 8% polyacrylamide/urea sequencing gels. Radiolabeled products were detected by autoradiography using Kodak XAR5 or Cronex x-ray film at −70°C with intensifying screens for 24–72 hr.

DNase I digestions of deproteinized genomic DNA (10–15 μg) were performed as described (15) and analyzed by primer-extensions as described above. DNase I cleavage sites were mapped by dideoxynucleotide chain-termination sequencing (17) using undigested genomic DNA as template. The sequencing reactions were performed as described above for primer-extensions but with substitutions for dNTPs of a 10× ddNTP solution containing 3 mM dATP, dCTP, dGTP, and dTTP and 0.3 mM ddNTP.

The ^{32}P-labeled (18) oligonucleotide primers used to probe UAS_{CUP1} are shown diagrammatically in Fig. 1. Primer 1 hybridizes to sequences in the *CUP1* promoter from −248 to −233 (with respect to the transcription initiation site at +1)

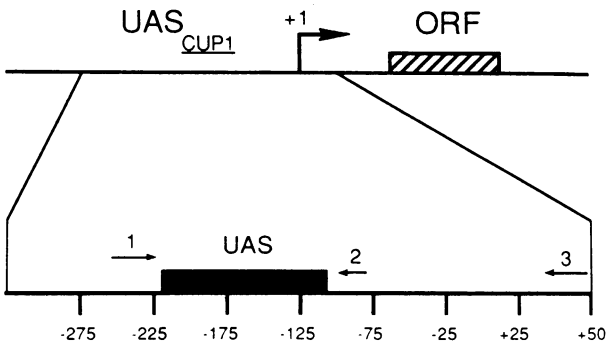

FIG. 1. Schematic diagram of the *CUP1* gene and the strategy employed for chromosomal footprinting. ORF indicates the open reading frame that encodes the yeast MT protein. UAS_{CUP1} indicates the UAS essential for copper inducibility of *CUP1* transcription (8). The large arrow (+1) represents the 5'-most start site for *CUP1* transcription. The oligonucleotide primers 1, 2, and 3 used for chromosomal footprinting are shown with the site of hybridization relative to UAS_{CUP1}. The arrows representing the oligonucleotides point to the direction of primer-extension.

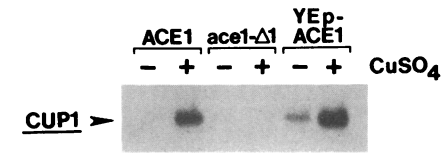

FIG. 2. *ACE1* gene dosage effects on *CUP1* transcription *in vivo*. Total RNA from strains DTY22 (*ACE1*), DTY26 (*ace1-Δ1*), and H9/YEp-ACE1 was isolated from uninduced (−) and copper-induced (+) cultures. Steady-state levels of *CUP1* mRNA were analyzed by RNA blotting and hybridization with a ^{32}P-labeled *CUP1 Xba* I/*Kpn* I restriction fragment. The arrowhead indicates the position of *CUP1* gene-specific mRNA.

and was used to analyze DNase I cleavage products derived from the lower strand. Primers 2 and 3 hybridize to positions −83 to −107 and +49 to +33, respectively, and were used for the analysis of upper (sense) strand DNase I cleavage products.

RESULTS

***ACE1* Gene Dosage Effects on *CUP1* Transcription.** It was recently demonstrated that a functional *ACE1* gene is essential for copper-induced transcription of the *CUP1* gene (9). Because *ACE1* exerts its effect via UAS_{CUP1} (M. Szczypka and D.J.T., published data), we tested the effects of multiple copies of the cloned *ACE1* gene on *CUP1* gene transcription. Fig. 2 shows an RNA blot analysis of total cell RNA probed with a ^{32}P-labeled *CUP1/Xba* I/*Kpn* I 0.7-kb DNA restriction fragment that spans the *CUP1* structural gene. The wild-type strain DTY22 exhibited an ≈10-fold induction of steady-state levels of *CUP1* mRNA after copper treatment. The isogenic *ace1-Δ1* strain DTY26 displayed no apparent induction of *CUP1* mRNA levels, consistent with previous analyses of an integrated *CUP1–lacZ* fusion in this same strain (9). The introduction of the *ACE1* gene on the high copy-number, 2-μm-based plasmid YEp13 (11) in strain H9 resulted in an ≈3-fold increase in basal and copper-induced steady-state levels of *CUP1* mRNA relative to those of strain DTY22. H9 harboring plasmid YEp13 alone gave steady-state levels of uninduced and copper-induced *CUP1* mRNA indistinguishable from those of DTY22 (data not shown). The *ACE1* gene product may therefore be present in limiting amounts in wild-type yeast strains.

Chromosomal Footprinting of the *CUP1* Promoter. It is clear from the analysis of several yeast promoters that UAS regions serve as binding sites for cellular factors that regulate gene transcription (19). We therefore carried out chromosomal footprinting analyses to determine whether factors bind to UAS_{CUP1} and, if present, to ascertain whether the *ACE1* gene plays a role in the occupation of UAS_{CUP1} by cellular factors. The chromosomal footprinting technique utilizes high-resolution analysis of DNase I partial cleavage products from DNA–protein complexes present in yeast lysates (15). Cleavage products are analyzed by extension of 5'-end-labeled oligonucleotide primers that hybridize at a specific site relative to the region of interest. Extension of the primer to the site of DNase I cleavage, followed by resolution of the extension products on sequencing gels, maps the location of DNase I cleavages to single-nucleotide resolution. Our strategy for chromosomal footprinting of the *CUP1* promoter employed isogenic strains with different *ACE1* genotypes and each harboring three copies of the 2-kb *CUP1* repeat unit (5). Because basal and copper-induced levels of *CUP1* expression increase linearly with *CUP1* gene copy number (20), it is likely that these repeat units are subject to the same transcriptional regulatory controls. Precedent for this approach is given in the analysis of protein–DNA interactions at the metal regulatory elements of the rat MT

promoter from a cell line containing a 5- to 6-fold amplification of the MT genes (21).

Fig. 1 diagrammatically summarizes the organization of the *CUP1* gene and the location of UAS_{CUP1} between positions −220 and −105 with respect to the transcription initiation site (8). Each of the oligonucleotide primers used to analyze DNase I cleavage products is complementary to a single sequence in each *CUP1* repeat unit.

Fig. 3 shows the chromosomal DNase I footprint analysis of the top (sense) strand of the UAS_{CUP1} region using primer 2. Lane U contains primer-extension products of yeast genomic DNA that has not been treated with DNase I. This provides a control for background due to either endogenous yeast nucleases or primer-extension artifacts. The DNase I digestion pattern of deproteinized yeast genomic DNA is shown in lanes N_1, N_2, and N_3. Genomic sequencing lanes (A and C) were used to map the sites of DNase I cleavages. Footprinting analysis of cell lysates from the wild-type strain DTY22 (*ACE1*) revealed protection from DNase I cleavage within UAS_{CUP1}. This protection was copper-inducible and extends from −121 to −144 with respect to the *CUP1* transcription initiation site denoted +1 (6) (Fig. 3, lanes ACE1 − and +). Sites of enhanced DNase I cleavage also occur in samples from copper-induced DTY22 cells at positions −145 and −146 and further upstream between positions −219 and −227. Samples from strain DTY22 (*ace1-Δ1*) displayed no obvious differences in DNase I cleavage pattern between uninduced and copper-induced cells (Fig. 3, lanes ace1-Δ1 − and +). The location and relative frequency of DNase I cleavage products were indistinguishable from those observed for the uninduced wild-type strain DTY22 (Fig. 3, lane ACE1 −). We observed two major differences between UAS_{CUP1} footprints from strains DTY22 and H9/YEp-ACE1. (*i*) The sequence protected from DNase I cleavage in copper-induced DTY22 (−121 to −144) was more dramatically protected in H9/YEp-ACE1, and the protected region was more extensive, from −114 to −144 (Fig. 3, lanes YEp-ACE1 − and +). (*ii*) We observed two other regions of UAS_{CUP1} that were protected from DNase I cleavage in copper-induced H9/YEp-ACE1 cells. One region extends from −163 to −188 and the other extends from −195 to −202. These additional protected sequences are dependent upon multiple copies of the *ACE1* gene since the YEp13 plasmid alone in H9 gave rise to a copper-inducible footprint of UAS_{CUP1} indistinguishable from that of DTY22 (data not shown).

An additional copper-dependent change in DNase I cleavage is observed in the region from −230 to −290 in the wild-type *ACE1* strain DTY22 and H9/YEp-ACE1 but not in the strain DTY26 (*ace1-Δ1*). DNase I cleavages within this region are enhanced in samples from copper-induced DTY22 and H9/YEp-ACE1 cells (Fig. 3). Whether these enhanced cleavages are due to factor binding to downstream UAS_{CUP1} sequences or are a result of interactions of cellular factors with DNA sequences between −230 and −290 is unclear. This region is near the 3′ end of a transcriptional unit flanking the *CUP1* gene (6). We also note that comparison of the DNase I cleavage products of deproteinized genomic DNA (Fig. 3, lanes N_1, N_2, and N_3) with those from cell lysates from the yeast strains used here reveals some differences in the overall sensitivity to DNase I cleavage. These differences may be chromatin-specific and their cause is unknown. The DNase I cleavage patterns of deproteinized DNA from uninduced and copper-induced cells are indistinguishable (data not shown).

Primer 1 was used to analyze the bottom strand of UAS_{CUP1} to verify the copper-induced changes in DNase I cleavage patterns observed with primer 2 on the sense strand. For samples from strain DTY22 (*ACE1*), protection from DNase I cleavage was observed between positions −119 and −146 (Fig. 4, lanes ACE1 − and +). No obvious copper-induced enhanced DNase I cleavages were observed in these samples. As with primer 2, we observed no differences in DNase I cleavage patterns from uninduced or copper-induced DTY26 (*ace1-Δ1*) cell lysates (Fig. 4, lanes ace1-Δ1 − and +). Footprint analysis of samples from H9/YEp-ACE1

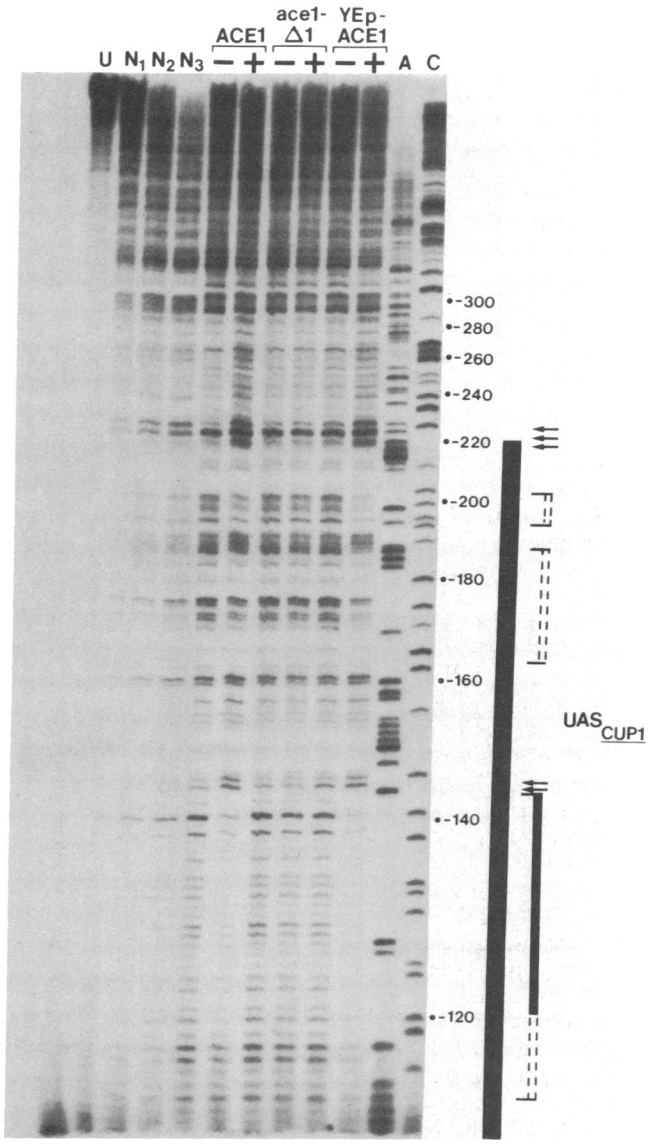

FIG. 3. Chromosomal footprinting of UAS_{CUP1} with primer 2. Chromosomal footprinting was carried out on *CUP1* promoter sequences from the following uninduced (−) and copper-induced (+) cell cultures: DTY22 (*ACE1*), DTY26(*ace1-Δ1*), and H9/YEp-ACE1. The products of DNase I cleavage were analyzed by hybridizing and extending ^{32}P-labeled primer 2 and fractionating the extension products on an 8% polyacrylamide/urea sequencing gel. Control reactions are as follows: lane U, undigested genomic DNA; lanes N_1, N_2, and N_3, deproteinized yeast DNA treated with increasing concentrations of DNase I; lanes A and C, dideoxynucleotide chain-termination sequencing reactions of the *CUP1* promoter region using primer 2 with chromosomal DNA from strain DTY22. The heavy vertical filled black bar to the right of the panels (here and in Figs. 4 and 5) indicates the extent of UAS_{CUP1}. Thin vertical filled bars indicate regions of protection from DNase I cleavage observed in DTY22 (*ACE1*) and H9/YEp-ACE1 samples. Dashed vertical bars indicate regions protected from DNase I cleavage only detected in samples from H9/YEp-ACE1. Regions of enhanced DNase I cleavage are indicated with arrows. Nucleotide positions relative to the transcription initiation site (+1) are indicated to the right of the sequencing lanes.

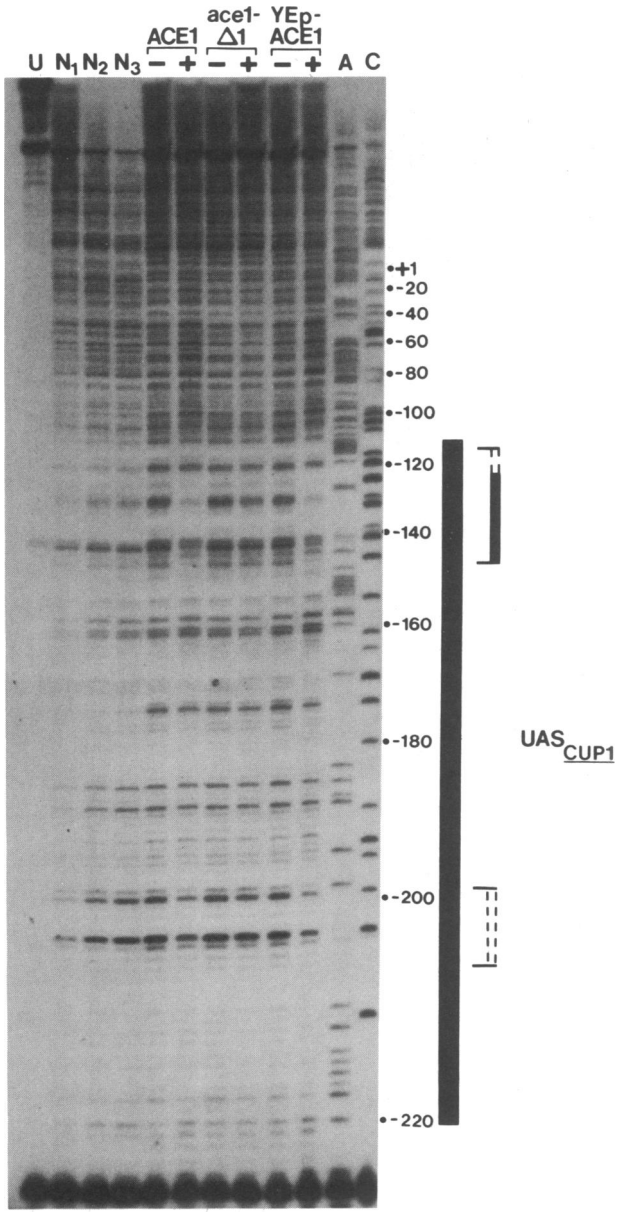

FIG. 4. Chromosomal footprinting analysis of the *CUP1* promoter region using primer 1. DNase I cleavage products from uninduced (−) and copper-induced (+) cell cultures were analyzed from strains DTY22 (*ACE1*), DTY26 (*ace1-Δ1*), and H9/YEp-ACE1. DNase I cleavage products were analyzed by primer-extension reactions using ^{32}P-labeled primer 1.

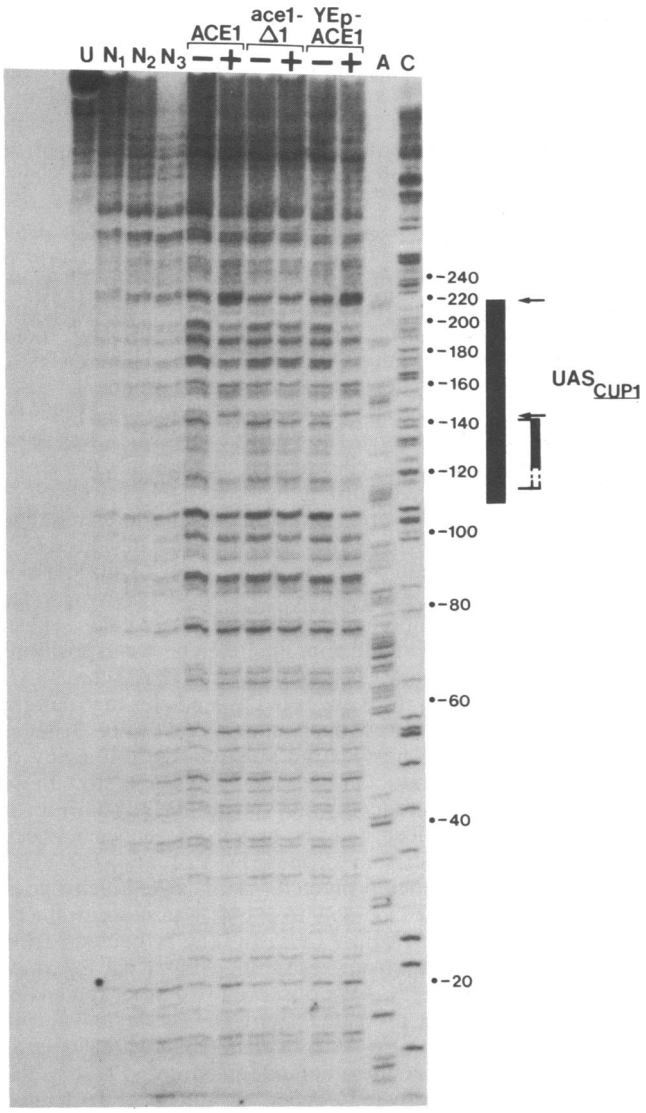

FIG. 5. Footprinting downstream of UAS_{CUP1}. DNase I cleavage products from the strains used in Fig. 4 were analyzed by primer-extension reactions using ^{32}P-labeled primer 3. Control reactions are indicated by the heavy vertical filled bar. A region of enhanced DNase I cleavage is indicated by an arrow. Thin vertical filled bars indicate regions of protection from DNase I cleavage observed in DTY22 (*ACE1*) and H9/YEp-ACE1. Dashed vertical bars indicate regions protected from DNase I cleavage only detected in samples from H9/YEp-ACE1.

revealed copper-induced protection from DNase I cleavage from positions −112 to −146 and from −198 to −206 (Fig. 4, lanes YEp-ACE1 − and +). No protection was observed on this strand between residues −163 and −188. This region was observed to be protected on the top strand using primer 2. Cleavages specific to chromatin samples were also observed on this strand between −160 and −180 (Fig. 4, lanes N_1, N_2, and N_3).

Transcription of yeast genes by RNA polymerase II involves other promoter elements such as TATA regions and sequences that influence the choice of transcription start sites (19). Sequences resembling TATA elements are found centered at approximately −30 and −75 in the *CUP1* promoter (6). We used primer 3 to analyze the region downstream of UAS_{CUP1} (Fig. 5). We observed copper-inducible protection of UAS_{CUP1} sequences consistent with those observed at higher resolution using primer 2; however, no other differences were apparent between deproteinized DNA and chromatin in the −8 to −110 region under any conditions.

DISCUSSION

Our results demonstrate induced occupation of UAS_{CUP1} sequences in copper-treated wild-type cells relative to cultures grown in the absence of exogenous copper. This copper-inducible occupation does not occur at detectable levels in cells bearing a nonfunctional *ACE1* locus and occurs more extensively and at additional locations in UAS_{CUP1} in cells harboring the *ACE1* gene on a multicopy yeast plasmid. A summary of UAS_{CUP1} sequences protected from DNase I cleavage and sites of enhanced DNase I cleavage for strains DTY22 (*ACE1*) and H9/YEp-ACE1 is shown in Fig. 6. The identity of the cellular factor(s) that effects the copper-inducible UAS_{CUP1} footprints is unknown. The observation that a functional *ACE1* gene is necessary for their binding to

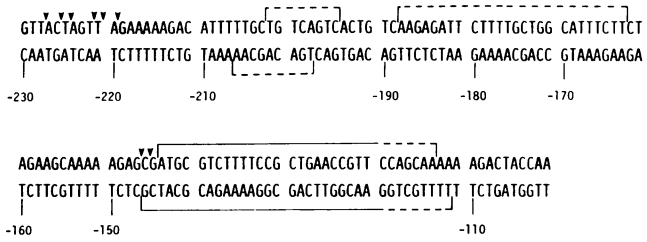

FIG. 6. Summary of UAS_{CUP1} chromosomal footprinting results. The sequence of the *CUP1* promoter from −101 to −230 is shown, with the top sequence representing the sense strand. Solid brackets indicate sequences protected from DNase I cleavage observed in copper-induced DTY22 (*ACE1*) and H9/YEp-ACE1 strains. Dashed brackets indicate sequences protected from DNase I cleavage that are detectable only in samples from H9/YEp-ACE1. Arrowheads indicate sites of enhanced cleavage by DNase I.

UAS_{CUP1} suggests at least two possibilities. (*i*) The *ACE1* gene product may be a direct activator of *CUP1* gene transcription and bind to UAS_{CUP1} either alone or as a complex with other, as yet unidentified, factors. (*ii*) *ACE1* gene product may regulate the biosynthesis or activity of other cellular factor(s) that directly bind to UAS_{CUP1} sequences.

The three subregions of UAS_{CUP1} protected from DNase I cleavage were not equally protected in these experiments. The most obvious copper-inducible protection from DNase I cleavage occurs between −120 and −144 on the top strand and between −120 and −146 on the bottom strand of UAS_{CUP1} in DTY22 (*ACE1* wild-type) cells. This protection was more extensive in H9/YEp-ACE1 cells, where it extended from −114 to −144 on the top strand and from −112 to −146 on the bottom strand. Two other subregions of UAS_{CUP1} were protected from DNase I cleavage only in copper-induced H9/YEp-ACE1 samples. Protection from DNase I cleavage on the top strand was observed between positions −195 and −202 and on the bottom strand from −198 to −206. We did not observe protection on the bottom strand of UAS_{CUP1}, which overlaps the top strand protection observed between positions −163 and −188. Although the reason for this is not clear, it might reflect an inherent resistance of this local sequence to DNase I cleavage since we observed few DNase I cleavages surrounding this site on this strand.

The differential occupation of three sites in UAS_{CUP1} with wild-type or elevated levels of *ACE1* suggests that a single transcription factor might bind to all three sites, but with different affinities. We note that the region, between −107 and −140, contains an inverted repeat centered between nucleotide positions −123 and −124. Within this inverted repeat lies two copies of the consensus 5′-GTCTTTTNNGCTG-3′ (−107 to −115 and −140 to −128). Only the upstream section of this subregion (−140 to −128) is fully protected in cells containing one copy of the *ACE1* gene, whereas the DNase I protection extends downstream to at least −112 in cells containing the *ACE1* gene on a multicopy plasmid. This extended protection may well represent binding of a factor as far downstream as −105, which would cover the downstream 5-GTCTTTTNNGCTG-3′ repeat; however, this region lacks sufficient DNase I cleavages to assess the occupation of this region. Similarly, a partial repeat of this motif, 5′-GTCTTTT-3′, lies upstream (from −211 to −217) and overlaps a region of DNase I protection that is fully induced only in cells carrying multiple copies of the *ACE1* gene. The lack of diagnostic DNase I cleavages over the motif prevents assignment of the motif itself as protected; however, copper-inducible enhanced cleavages from −219 to −227 suggest that this sequence might be occupied by a protein. The UAS_{CUP1} region least well protected from DNase I cleavage in H9/YEp-ACE1 cells (−163 to −188) contains a still poorer repeat of this motif, 5′-CTTTT-3′ (−176 to −180) or 5′-TTNNGCTG-3′ (−172 to −179). Other potential candidates for a consensus binding site can also be found in UAS_{CUP1}, most notably 5′-TTTTGCTG-3′. Given the similarity of this sequence to 5′-GTCTTTTNNGCTG-3′, it might represent a lower affinity site for the same factor. At present we are unable to discount the possibility that these secondary sites are occupied by one or more distinct factor(s) that are also influenced by the *ACE1* gene.

In general, this observation is analogous to the detection of DNA–protein interactions at all five metal regulatory elements of the rat and mouse MT promoters (21, 22). Additional work will be required to identify the factor(s) bound to the UAS_{CUP1} subregions and to distinguish between a direct or indirect role for the *ACE1* gene product in promoter binding and *CUP1* gene activation.

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