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A Cysteine-Rich Nuclear Protein Activates Yeast Metallothionein Gene Transcription

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The *ACE1* gene of the yeast *Saccharomyces cerevisiae* is required for copper-inducible transcription of the metallothionein gene (*CUP1*). The sequence of the cloned *ACE1* gene predicted an open reading frame for translation of a 225-amino-acid polypeptide. This polypeptide was characterized by an amino-terminal half rich in cysteine residues and positively charged amino acids. The arrangement of many of the 12 cysteines in the configuration Cys-X-Cys or Cys-X-X-Cys suggested that the *ACE1* protein may bind metal ions. The carboxyl-terminal half of the *ACE1* protein was devoid of cysteines but was highly acidic in nature. The ability of a bifunctional *ACE1*–β-galactosidase fusion protein to accumulate in yeast cell nuclei was consistent with the possibility that *ACE1* plays a direct role in the regulation of copper-inducible transcription of the yeast metallothionein gene.

Metal tolerance in the bakers' yeast *Saccharomyces cerevisiae* is mediated, in large part, by high-level expression of the metallothionein protein encoded by the *CUP1* gene (6, 8, 10, 13, 14, 17, 39, 40). Two distinct mechanisms account for synthesis of large quantities of metallothionein in yeast: *CUP1* gene amplification and transcription induction of *CUP1* in response to high environmental copper concentrations (9, 14, 15, 27, 39, 39, 42). Copper-inducible transcription of *CUP1* requires the presence of *cis*-acting upstream activation sequences (UAS_{CUP1}) located between approximately −105 and −230 with respect to the transcription initiation site (40). The ability of a synthetic oligonucleotide, representing a portion of UAS_{CUP1}, to activate a heterologous yeast promoter in a copper-inducible fashion suggests that UAS_{CUP1} activates transcription by positive regulation (40).

Yeast strains bearing a mutation in the *ACE1* (activation of *CUP1* expression) gene are hypersensitive to copper due to a defect in copper-inducible transcription of the *CUP1* gene (39). The inability of strains harboring the *ace1-1* mutation to induce transcription of episomal *CUP1* promoter fusions in a copper-dependent fashion indicates that *ACE1* is a *trans*-acting genetic element (39). We previously cloned the wild-type *ACE1* gene by in vivo complementation based on the ability of yeast genomic fragments to restore copper resistance to otherwise copper-sensitive cells bearing the *ace1-1* mutation (39). Genetic linkage analysis and chromosomal deletion experiments demonstrated that the *ACE1* clone represents the authentic *ACE1* locus, which maps to chromosome VII, 9 centimorgans (cM) centromere distal to *lys5* (39). Although the precise role that the *ACE1* gene product plays in copper-inducible transcription of *CUP1* is unknown, chromosomal footprinting experiments have demonstrated that a functional *ACE1* gene is required for copper-inducible binding of a cellular factor(s) to UAS_{CUP1} (20a). Furthermore, increased *ACE1* gene dosage gives rise to increases in both basal and copper-induced levels of *CUP1* transcription and increases the number of UAS_{CUP1} sites occupied in copper-induced cells. These characteristics indicate that the *ACE1* gene product functions directly or indirectly with other yeast transcriptional components.

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Here we present the sequence of the cloned wild-type *ACE1* gene and examine its expression and function. The putative *ACE1* polypeptide, when fused to the *Escherichia coli* β-galactosidase protein, concentrates in the yeast cell nucleus. The data presented in this report are consistent with the *ACE1* gene product playing a direct role in copper-inducible transcription of the yeast *CUP1* gene and suggest that *ACE1* protein is activated posttranslationally as a consequence of high environmental copper concentrations.

MATERIALS AND METHODS

Yeast and bacterial strains and cell growth. The following yeast strains, all described previously (39), were used in these studies: H9 (*MATα his6 leu2 ura3-52 CUP1*$^{R-3}$), DTY20 (*MATα leu2 ura3-52 his ace1-1 CUP1*$^{R-3}$), DTY22 (*MATα his6 ura3-52 LEU2*::YipCL *CUP1*$^{R-3}$), and DTY26 (*MATα his6 LEU2*::YipCL *ace1-Δ1*::*URA3 CUP1*$^{R-3}$). All yeast strains were grown at 30°C in rich (YPD) medium or on synthetic complete (SC) medium lacking nutrients as specified before (37). Copper inductions were carried out by the addition of $CuSO_4$ to a final concentration of 100 μM, followed by incubation of cell cultures at 30°C and 300 rpm for 45 min. The *E. coli* strains DH5α F′ and GM33 were used for all DNA manipulations and were grown under standard conditions (31).

Plasmids. Plasmids containing subclones of the genomic fragment encompassing the wild-type *ACE1* gene (see Fig. 1) were constructed by using DNA restriction fragments from the 3.2-kilobase (kb) *Eco*RI fragment in pRI-3 (39). All standard DNA manipulations were carried out as described before (31). A frameshift mutation in the *ACE1* open reading frame 225 (ORF-225) was created by filling in the unique *Bcl*I site contained within the 3.2-kb *Eco*RI DNA restriction fragment (in plasmid pRI-3) with the Klenow fragment of DNA polymerase I and ligating to recircularize the plasmid. The resultant plasmid, designated pMS114, harbors a 4-base-pair (bp) insertion at the former *Bcl*I site which terminates the *ACE1* ORF-225 14 codons downstream from the site of insertion. Plasmid p4d is composed of the 1-kb *ACE1* *Bgl*II DNA restriction fragment inserted into the *Bam*HI site of the centromere-based low-copy-number plasmid YCp50 (24). Plasmid p2g contains a 1.8-kb *Hin*dIII DNA restriction fragment rendered blunt with the Klenow fragment of DNA

polymerase I and inserted into the *Sma*I site of the centromere-based low-copy-number plasmid pTI-15 (kindly provided by T. Icho and R. Wickner). Plasmid pMS117 was constructed by insertion of a 944-bp *Ssp*I-*Hin*fI DNA restriction fragment rendered blunt with the Klenow fragment of DNA polymerase I into the *Sma*I site of pTI-15. Plasmids used to make *ACE1* gene strand-specific RNA probes were constructed by insertion of the 1.8-kb *ACE1* *Hin*dIII DNA restriction fragment into pT7-2 (U.S. Biochemicals) in both orientations with respect to the phage T7 promoter. Plasmid YEp-ACE1 was constructed by insertion of the 1.8-kb *Hin*dIII fragment into the high-copy-number yeast plasmid YEp13 (7). A translational fusion between the *ACE1* gene and the *E. coli* β-galactosidase (*lacZ*) gene was constructed by standard in vitro DNA manipulations (31). An 819-bp *Ssp*I-*Alu*I partial *ACE1* DNA restriction fragment was isolated by agarose gel electrophoresis and electroelution. This fragment was inserted into the *Sma*I site of plasmid pMC1871 (11) upstream from and in frame with the *E. coli* β-galactosidase (*lacZ*) gene. The fusion junction was sequenced by dideoxy-nucleotide chain termination sequencing (36) to verify that the expected fusion junction was in frame. The resultant plasmid, pMS115, was partially cleaved with *Bam*HI, and the agarose gel-purified 3.8-kb *Bam*HI *ACE1-lacZ* fragment was inserted into the *Bam*HI site of the high-copy-number yeast plasmid YEp13 to yield plasmid pMS116.

***ace1-1* complementation testing.** Subclones of the 3.2-kb *Eco*RI DNA restriction fragment (containing the wild-type *ACE1* gene) were introduced, by transformation (21) into yeast strain DTY23 (*ace1-1*) and tested for complementation. Complementation of the *ace1-1* mutation is assessed in two ways: restoration of copper resistance and restoration of the ability of these cells to transcriptionally induce a chromosomally integrated *CUP1-lacZ* fusion after exposure to copper sulfate (39). For all copper resistance tests, purified transformants and controls were streaked on SC agar and SC agar containing from 0 to 500 μM copper sulfate.

Sequence analysis of the *ACE1* locus. A cloned DNA restriction fragment harboring the *ACE1* gene was isolated from plasmid pRI-3 (39) as a 3.2-kb *Eco*RI fragment. This fragment was subcloned into the *Eco*RI site of M13mp18 in both orientations (33), and a nested set of closely spaced deletions was created by using exonuclease III as described before (20). Templates were sequenced on both strands with Sequenase enzyme (U.S. Biochemicals) in dideoxynucleotide chain termination sequencing reactions (36). The products of sequencing reactions were resolved on buffer gradient polyacrylamide-urea sequencing gels (4) and exposed to Kodak XAR-5 X-ray film. DNA sequences were compiled and analyzed with the MicroGenie program (Beckman).

RNA analyses. Total RNA was isolated from 1.5 ml of log-phase (OD_{650}, 1.0 to 1.5) untreated or copper-treated yeast cultures as described before (19). For RNA blot analysis, 20 μg of each RNA sample was fractionated on a 1.5% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probes (31). To detect *CUP1* gene-specific mRNA, a nick-translated *CUP1* *Xba*I-*Kpn*I DNA restriction fragment was used (41). *ACE1* mRNA was detected by using a nick-translated 1.8-kb *Hin*dIII DNA fragment purified from plasmid p2g by agarose gel electrophoresis. For *ACE1* strand polarity determinations, ^{32}P-labeled RNA probes were synthesized from phage T7 promoter plasmids harboring the 1.8-kb *ACE1* *Hin*dIII fragment in both orientations. Radioactive RNA probe synthesis (32) was carried out in the presence of [α-^{32}P]UTP in a reaction catalyzed by bacteriophage T7 RNA polymerase (a gift from J. Y. Lee and D. R. Engelke). The size of the RNA species homologous to the 1.8-kb *ACE1* *Hin*dIII DNA restriction fragment was estimated by using ^{32}P-labeled RNA size markers synthesized in vitro from templates pT7-0 (U.S. Biochemicals) and Riboprobe positive control (Promega) with [α-^{32}P]UTP and bacteriophage T7 RNA polymerase. Radioactive RNA markers were cofractionated on 1.5% agarose–formaldehyde gels, transferred to nitrocellulose, and exposed to X-ray film with the blots which had been incubated with probes and washed. Blots were exposed to X-AR5 X-ray film at −70°C with an intensifying screen. Yeast *PYK1* mRNA was detected by using a nick-translated plasmid containing the *PYK1* gene and served as a control for the amount and integrity of RNA loaded onto each lane of all RNA gels.

Primer extension analysis of *ACE1* gene-specific mRNA was carried out with 25 μg of total RNA from untreated or copper-treated cell cultures by using avian myeloblastosis virus reverse transcriptase. The ^{32}P-, 5′-end-labeled oligonucleotide primer used was a 16-mer corresponding to nucleotide positions 224 to 239 in Fig. 2, synthesized on an Applied Biosystems 380A DNA synthesizer and 5′-end labeled with ^{32}P as described before (31). The radiolabeled products of primer extension reactions were fractionated on 8% polyacrylamide–urea sequencing gels.

β-Galactosidase assays. β-Galactosidase activity was assayed (16) in yeast cell cultures grown to log phase (OD_{650}, 1.0 to 1.5) and untreated or treated with 100 μM $CuSO_4$ for 45 min. Units of β-galactosidase activity were normalized to cell culture density. All assays were carried out at least three times, and enzyme activity values differed by less than 20%.

Indirect immunofluorescence analyses. Yeast cells from 10-ml cultures grown to log phase (OD_{650}, 1.0) in SC medium lacking leucine were prepared for indirect immunofluorescence as described before (1, 28). Affinity-purified mouse monoclonal antibody against *E. coli* β-galactosidase (Promega) was used at working concentrations of between 20 and 80 μg/ml. Sheep anti-mouse immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC) was purchased from Boehringer Mannheim and used at a concentration of 30 μg/ml. Yeast cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), a gift of B. Harrer. Cells were visualized with a 100× objective lens with a Leitz Orthoplan microscope equipped for epifluorescence and phase-contrast microscopy. Cells were photographed with Kodak T-Max (asa 400) film.

RESULTS

Less than 1 kb of yeast DNA is sufficient for complementation of the *ace1-1* mutation. We previously demonstrated that a 2.8-kb yeast genomic DNA fragment complements both the copper sensitivity and *CUP1* transcription induction defects in a yeast strain bearing the *ace1-1* mutation (39). To more precisely localize *ACE1* gene activity, we constructed subclones of this fragment in the centromere-based low-copy-number yeast plasmid pTI-15. These derivatives were introduced by transformation (21) into yeast strain DTY23 (*ace1-1*) and tested for complementation of the *ace1-1* mutation. Strain DTY23 harbors three copies of the *CUP1* amplification unit (39). Therefore, restoration of *ACE1* gene activity is measured by the ability of plasmid recipients to resist concentrations of $CuSO_4$ up to 500 μM. Furthermore, complementation of the *ace1-1* mutation in this strain transcriptionally activates a *CUP1-lacZ* fusion, integrated at

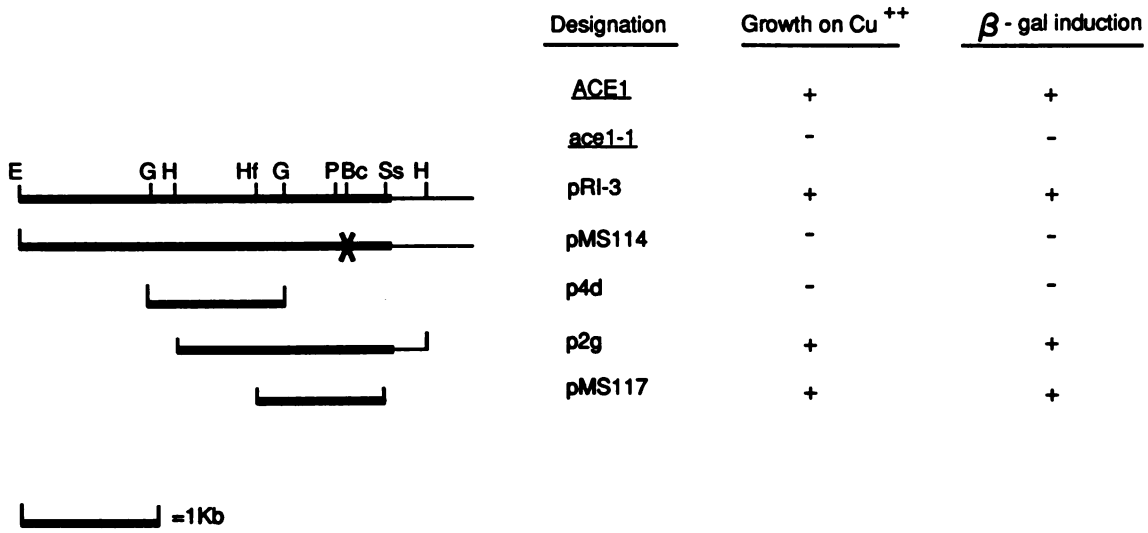

FIG. 1. Subcloning analysis of the wild-type *ACE1* locus. Subclones of the wild-type *ACE1* locus in plasmid pRI-3 were constructed in the low-copy-number centromere-based plasmid pTI-15 and introduced by transformation into strain DTY23 bearing the *ace1-1* mutation. Complementation was ascertained by streaking transformants to SC medium and SC plates containing a range of copper sulfate concentrations to determine levels of copper resistance. Restoration of copper-inducible transcription was determined by β-galactosidase (B-gal) assays of untreated and copper-treated transformant cultures and compared with the wild-type control transformant DTY22(pTI-15). Symbols: +, complementation to wild-type *ACE1* levels; −, no complementation. Thick lines correspond to yeast DNA sequences, and thin lines correspond to vector sequences. The X indicates the site of the 4-bp insertion in plasmid pMS114. Abbreviations: E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; Hf, *Hin*fI; P, *Pvu*II; Bc, *Bcl*I; Ss, *Ssp*I.

leu2, in a copper-inducible fashion (39). Figure 1 shows the results of *ace1-1* mutation complementation assays obtained after introducing subclones of the 2.8-kb *ACE1* restriction fragment into strain DTY23. These data demonstrate that a yeast genomic DNA fragment of 0.95 kb, contained on plasmid pMS117, was sufficient to fully complement the *ace1-1* mutation to wild-type levels. Plasmid p4d, which contains a 1-kb *Bgl*II DNA restriction fragment that was present in all wild-type *ACE1* clones originally isolated (39), failed to complement the *ace1-1* mutation even partially. This result, coupled with our previous observation that this 1-kb *Bgl*II fragment from the *ACE1* locus is necessary for *ACE1* gene activity (39), indicates that this fragment is required but not sufficient for *ACE1* gene function.

To test for the possible presence of an open reading frame (ORF) for translation in the functional region of the *ACE1* DNA fragment, we introduced a 4-bp insertion at the unique *Bcl*I site in the *ACE1* DNA fragment in plasmid pRI-3. The resultant plasmid, pMS114, failed to complement the *ace1-1* mutation in strain DTY23, as ascertained by copper resistance testing and by analyzing copper-inducible expression of the integrated *CUP1-lacZ* fusion (Fig. 1). This result is consistent with the possibility that the *Bcl*I restriction site lies within an ORF for translation which is necessary for *ACE1* gene expression or function.

Sequence of the *ACE1* gene. To begin to characterize in detail the important structural and functional features of the *ACE1* gene and its encoded product, we determined the strand polarity of the gene by using strand-specific RNA probes. The 1.8-kb *ACE1 Hin*dIII DNA restriction fragment from plasmid p2g was subcloned into the *Hin*dIII site of the phage T7 promoter vector pT7-2 in both orientations. Radioactive [^{32}P]RNA probes representing each strand were synthesized from DNA templates (linearized 3′ to inserted *ACE1* sequences) in a reaction catalyzed by phage T7 RNA polymerase. The strand-specific radioactive RNA probes were independently hybridized to nitrocellulose strips containing agarose-formaldehyde gel-fractionated RNA from yeast strain DTY22. The results of this experiment revealed that only one strand-specific probe hybridized to an approximately 0.85-kb mRNA species, which was the only RNA species detected with either probe (data not shown). These results identified the putative coding strand of the *ACE1* gene.

The DNA sequence encompassing the 1.8-kb *ACE1 Hin*dIII DNA restriction fragment on the coding strand was determined by dideoxynucleotide chain termination sequencing as described in Materials and Methods. Because the 0.95-kb insert in plasmid pMS117 fully complemented the *ace1-1* mutation, we present here the sequence of the 1,100 bp of the cloned *ACE1* locus within which this fragment was contained (Fig. 2). Computer analysis of this DNA sequence indicated the presence of a single significant ORF on the *ACE1* gene sense strand. The longest ORF was initiated with an ATG triplet located at nucleotide position 195, with a second potential translation initiation codon located at position 288 and in frame with the upstream ATG. Although no evidence exists for the use of either of these ATG codons for translation, the presence of purine residues at positions 192 and 198 (−3 and +4 with respect to the ATG at position 195) suggests that this ATG may be preferentially utilized for translation initiation (29).

The ORF located between nucleotide residues 195 and 869, as deduced from the DNA sequence, had the potential to encode a polypeptide of 225 amino acid residues with a predicted molecular weight of 24,428. This putative *ACE1* gene product had an unusual amino acid composition and arrangement (Fig. 2). All 12 cysteine residues encoded by ORF-225 lay within the amino-terminal 105 amino acid residues. Furthermore, of a total of 29 basic residues (lysine or arginine) encoded by ORF-225, 25 resided within the amino-terminal 108 amino acid residues. The carboxy-terminal 117 residues contained only four basic residues. This

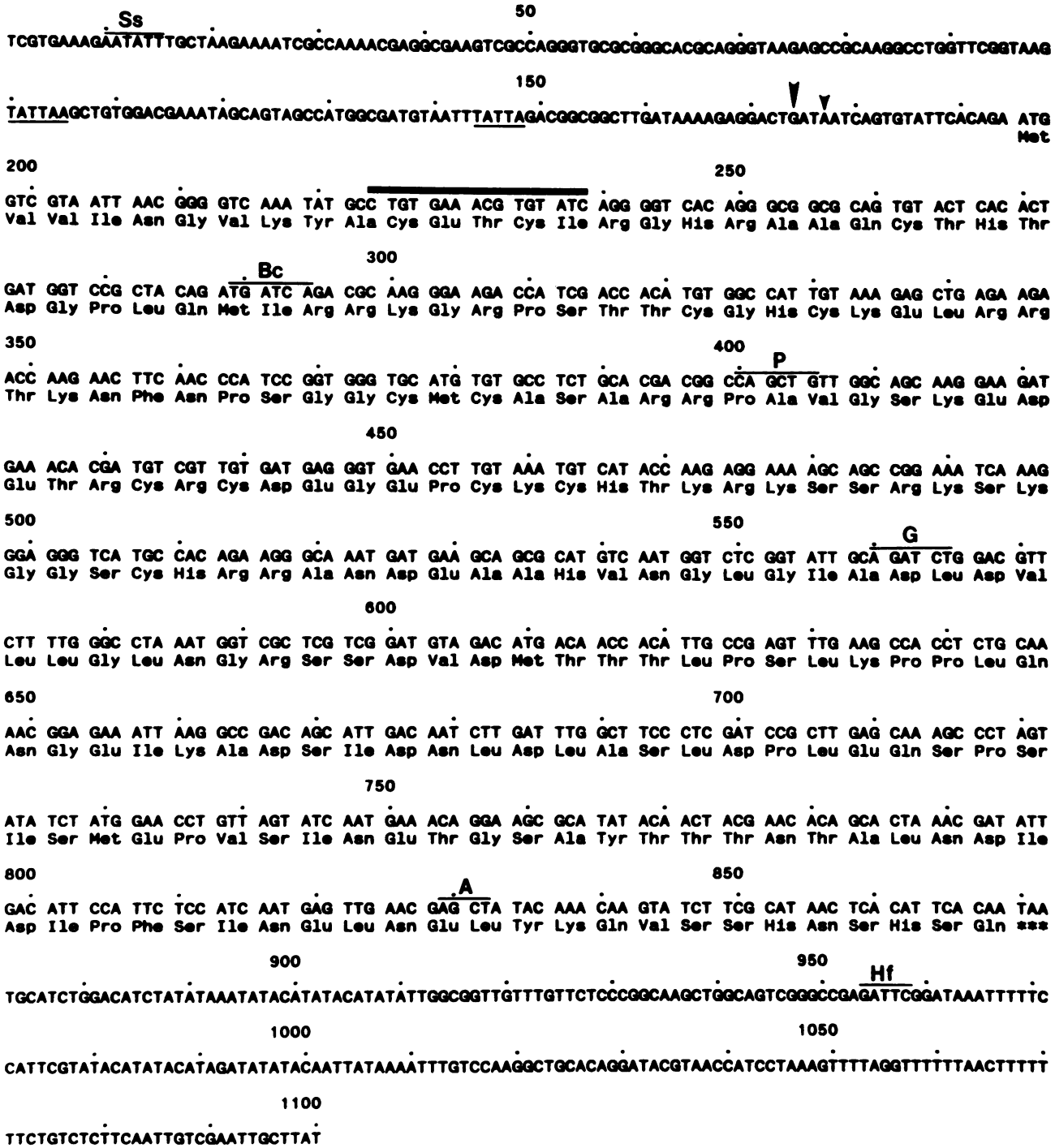

FIG. 2. Nucleotide sequence of the *ACE1* locus. The sequence of 1,100 nucleotides of the cloned *ACE1* gene sense strand is shown with the deduced amino acid sequence of the putative *ACE1* polypeptide. The longest potential ORF for translation (ORF-225) begins with the ATG at nucleotide position 195 and terminates at the TAA triplet at nucleotide position 870 (indicated by asterisks). The major (large arrowhead) and minor (small arrowhead) primer extension products are shown 5′ to the beginning of ORF-225. Two TATA-like elements 5′ to ORF-225 are underlined. Nucleotide residues complementary to the probe used for primer extension reactions are overlined with a heavy black line. Restriction enzyme sites are overlined and abbreviated: Ss, *Ssp*I; Bc, *Bcl*I; P, *Pvu*II; G, *Bgl*II; A, *Alu*I; Hf, *Hin*fI.

same region contained 18 of the 25 acidic amino acid residues (aspartic or glutamic acid) encoded in ORF-225.

The DNA sequences located upstream and downstream of ORF-225 encoded no extended ORFs for translation. Two stretches of nucleotide sequences upstream of ORF-225 did show similarity to the TATA box elements known to play an important role in gene transcription. The sequences resembling TATA elements were located beginning at nucleotide positions 100 (TATTAA) and 144 (TATTA). We currently have no direct evidence for a role of these sequences in the transcription of DNA sequences encompassing ORF-225; however, their positions upstream of the *ACE1* transcription

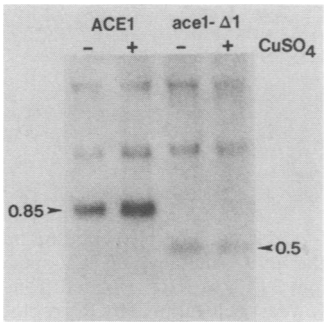

FIG. 3. Analysis of *ACE1*-specific RNA in wild-type and *ace1-Δ1* cells. Total RNA (20 μg per lane) isolated from untreated (−) or copper-treated (+) cultures of isogenic strains DTY22 (*ACE1*) and DTY26 (*ace1-Δ1*) was fractionated on a 1.5% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled nick-translated 1.8-kb *ACE1 Hin*dIII DNA restriction fragment. The sizes of the *ACE1*-specific RNA species (in kilobases) are indicated adjacent to the RNA species with arrowheads. The upper two hybridizing RNA species in all four lanes represent nonspecific cross-hybridization to the 25 and 18S rRNAs and were not observed in other experiments.

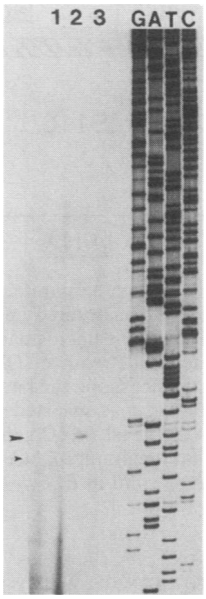

FIG. 4. Primer extension analysis of *ACE1*-specific RNA. Primer extension reactions with an oligonucleotide primer corresponding to *ACE1* antisense strand residues 224 to 239 were carried out in the presence of 20 μg of RNA from H9(YEp13) (lane 1), H9(YEp-ACE1) (lane 2), and 20 μg of calf liver tRNA (lane 3). The same oligonucleotide was used in dideoxynucleotide chain termination sequencing reactions with a single-stranded *ACE1* DNA template subcloned into M13 mp18 (lanes G, A, T, and C). Large and small arrowheads point to the major and minor primer extension products, respectively.

initiation site (see below) suggest that they may play a role in *ACE1* gene transcription.

Characterization of the *ACE1*-specific mRNA. To characterize the extent of the *ACE1* gene transcription unit, we carried out RNA blot analyses to determine the approximate size of the *ACE1* gene-specific RNA species. Using a nick-translated ^{32}P-labeled 1.8-kb *ACE1 Hin*dIII DNA restriction fragment isolated from plasmid p2g as a probe, we detected an approximately 0.85-kb RNA species from the wild-type strain DTY22 (Fig. 3). The abundance of this RNA species did not increase significantly after treatment of this strain with 100 μM $CuSO_4$ for 45 min. Hybridization with this probe to total RNA from untreated and copper-treated isogenic DTY26 (*ace1-Δ1*) cells revealed the presence of an approximately 0.5-kb RNA species and the absence of the 0.85-kb RNA species. The steady-state level of the 0.5-kb RNA species from strain DTY26 was also not appreciably altered by treatment of these cell cultures with copper sulfate. Because strain DTY26 has suffered deletion of a 1-kb *Bgl*II DNA fragment from the *ACE1* locus (39), the presence of the shorter RNA species in this strain is consistent with the possibility that the *ace1-Δ1* mutation has created a deletion in the *ACE1* gene transcription unit. Yeast cells harboring the *ace1-Δ1* allele fail to induce *CUP1* gene transcription (39) and lack a copper-inducible UAS_{CUP1}-binding activity (20a). These phenotypes could arise as a result of truncation of the *ACE1*-specific mRNA (and ORF-225) or may be a consequence of the lower steady-state levels of *ACE1* gene-specific mRNA in DTY26 (Fig. 3). Both possibilities may contribute to the mutant phenotype observed for this allele.

To determine the start site(s) for transcription of the *ACE1* gene, we carried out primer extension experiments with a ^{32}P-, 5′-end-labeled oligonucleotide primer corresponding to the *ACE1* minus strand from nucleotide residues 224 to 239 (Fig. 2). Experiments with wild-type strains with this approach yielded extension products visible only upon overexposure of autoradiograms (data not shown). We therefore analyzed RNA from strain H9 carrying YEp-ACE1, a high-copy-number yeast plasmid containing the 1.8-kb *ACE1 Hin*dIII DNA restriction fragment, and H9 harboring the vector YEp13 (7). The results of primer extension analyses with these strains are shown in Fig. 4 and summarized in Fig. 2. We detected one major and one minor extension product from H9(YEp-ACE1) RNA, which mapped to nucleotide positions 174 and 177, respectively (Fig. 4, lane 2). These correspond to primer extension products observed in lane 1 on overexposed autoradiograms and from RNA from H9 bearing no exogenous plasmid DNA (data not shown). The low level of steady-state *ACE1* transcripts observed by primer extension was consistent with results obtained by RNA blotting (Fig. 3) and similar to the observed low-level expression of other yeast transcription regulatory genes (5, 30, 35). The two primer extension products observed here mapped 21 and 18 nucleotides 5′ to the first ATG of the *ACE1* ORF-225 and 3′ to both TATA-like elements found in the region 5′ to ORF-225 (Fig. 2).

***ACE1-lacZ* fusion is functional in yeast cells.** The *ACE1* gene product functions to induce *CUP1* gene transcription upon exposure of yeast cells to high levels of exogenous copper (39). Because steady-state levels of *ACE1* mRNA are not induced by copper, we carried out experiments to determine whether biosynthesis of the *ACE1* gene product is regulated at the level of translation. We tagged the expression of *ACE1* ORF-225 by constructing an in-frame translational fusion between the *ACE1* DNA encoding ORF-225 and the *E. coli* β-galactosidase (*lacZ*) gene. An 819-bp *Ssp*I-*Alu*I *ACE1* DNA restriction fragment (nucleotide positions 12 to 830, Fig. 2) containing the amino-terminal 212 codons of ORF-225 was fused to codon 8 of the *E. coli lacZ* gene via a polylinker site in plasmid pMC1871 (11). This fusion was mobilized into the high-copy-number yeast vector YEp13 (7) to create plasmid pMS116 (Fig. 5). Plasmids pMS116 and YEp13 were introduced independently into yeast strains H9 (*ACE1*) and DTY20 (*ace1-1*) by transforma-

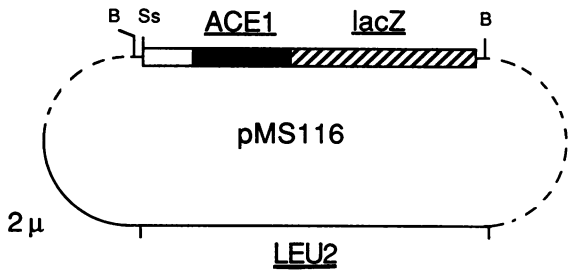

FIG. 5. Structure of the high-copy-number *ACE1-lacZ* gene fusion plasmid. A fusion was constructed between codon 212 of the *ACE1* ORF and the *E. coli* β-galactosidase gene. The open box indicates *ACE1* sequences upstream of ORF-225. The solid area indicates *ACE1* ORF sequences, and the hatched area indicates *lacZ* gene sequences. The fusion was inserted into the *Bam*HI site of YEp13, which contains the yeast *LEU2* gene, 2μm plasmid replication sequences (solid thin lines), and DNA sequences for selection and maintenance of the plasmid in *E. coli* (dashed lines). Abbreviations: B, *Bam*HI; Ss, *Ssp*I.

tion, and leucine prototrophs were selected and purified. Transformants were then assayed for β-galactosidase activity. β-Galactosidase activity was detected at low levels in both strains, and the level of activity detected was not significantly increased by treating transformant cultures with copper under conditions known to induce expression of a *CUP1-lacZ* fusion (Table 1). These results demonstrate that the *ACE1-lacZ* fusion protein is not significantly biosynthetically regulated by copper and suggest that the authentic *ACE1* protein also is not translationally regulated to a significant degree in response to copper. These data, in combination with *ACE1* gene-specific mRNA analyses, indicate that the *ACE1* gene product is constitutively expressed at a low level.

Cells carrying plasmid pMS116 were tested for copper resistance levels to determine whether the *ACE1-lacZ* fusion product was capable of complementing the *ace1-1* mutation. We compared the ability of the high-copy-number *ACE1-lacZ* fusion plasmid pMS116 and the high-copy-number YEp-ACE1 plasmid to render strain DTY20 copper resistant. Both plasmids complemented the *ace1-1* mutation by this analysis, rendering the recipient strain DTY20 resistant to equally high levels of copper (Table 1). We examined whether the *ACE1-lacZ* fusion on pMS116 restored copper-

TABLE 1. Expression and function of the *ACE1-lacZ* gene fusion

Strain	*ACE1* allele	Plasmid	β-Galactosidase activity[a] (U)		Growth on copper[b]
			−Cu	+Cu	
DTY7	*ACE1*	pMS116	2.4	2.6	+
		YEp13	ND[c]	ND	+
		YEp-ACE1	ND	ND	+
DTY20	*ace1-1*	pMS116	3.5	3.9	+
		YEp13	ND	ND	−
		YEp-ACE1	ND	ND	+

[a] β-Galactosidase activity was measured in untreated cells (−) or cells treated with 100 μM $CuSO_4$ for 45 min (+). Units of activity were normalized to cell culture density.

[b] Copper resistance was determined by streaking cells on SC medium and SC plus a range of copper sulfate concentrations. Symbols: +, growth on SC agar containing 500 μM $CuSO_4$; −, no growth.

[c] ND, None detected.

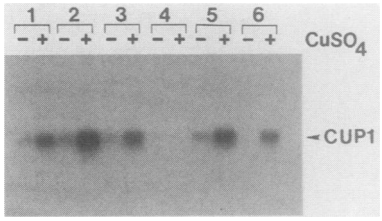

FIG. 6. *ACE1-lacZ* fusion complements the transcription defect in cells bearing the *ace1-1* mutation. *CUP1*-specific mRNA levels were analyzed from 20 μg of RNA isolated from untreated (−) or copper-treated (+) yeast cell cultures by RNA blotting. Each pair of lanes contains total RNA isolated from H9(YEp13) (lanes 1), H9(YEp-ACE1) (lanes 2), H9(pMS116) (lanes 3), DTY20(YEp13) (lanes 4), DTY20(YEp-ACE1) (lanes 5), and DTY20(pMS116) (lanes 6). The *CUP1*-specific RNA species is indicated.

inducible transcription to the chromosomal *CUP1* locus by blot hybridization to RNA isolated from untreated and copper-induced cultures. Strain DTY20 (*ace1-1*) harboring plasmid YEp13 exhibited no detectable copper-inducible transcription of *CUP1*, as previously observed for strains containing the *ace1-1* mutation (39) (Fig. 6, lanes 4). As expected, the high-copy-number plasmid carrying the authentic *ACE1* gene, YEp-ACE1, complemented the transcription defect in this strain, giving rise to a higher basal level of transcription and an approximately 10-fold induction in response to copper (Fig. 6, lanes 5). The introduction of the *ACE1-lacZ* fusion on pMS116 rendered *CUP1* transcription copper inducible in this strain, giving rise to somewhat lower basal and copper-induced levels than YEp-ACE1 (Fig. 6, lanes 6). This result confirmed our expectation, as initially determined by copper resistance testing, that the *ACE1-lacZ* fusion protein would complement the *ace1-1* mutation. Consistent with our previous observations, the introduction of YEp-ACE1 into an *ACE1* wild-type strain increased both basal and copper-induced levels of *CUP1* gene transcription three- to fivefold (Fig. 6, compare lanes in 1 and 2). This observation was not unexpected, since cells harboring plasmid YEp-ACE1 contain at least 10-fold higher *ACE1* mRNA levels than strains bearing a single copy of the *ACE1* gene (Fig. 4).

***ACE1*–β-galactosidase fusion protein accumulates in the yeast cell nucleus.** The ability of the *ACE1*–β-galactosidase fusion protein encoded on pMS116 to complement the *ace1-1* mutation suggests that the fusion protein is correctly localized in yeast cells. The detection of β-galactosidase activity in cells containing pMS116 implies that β-galactosidase expression occurs via fusion to the *ACE1* ORF-225 reading frame and thus serves as a tag for the intracellular location of the fusion protein. We analyzed DTY20 cells harboring pMS116 by indirect immunofluorescence for the presence and distribution of the *ACE1*–β-galactosidase fusion protein. Cells harboring pMS116 or the vector YEp13 alone were grown in SC medium lacking leucine to log phase and prepared for indirect immunofluorescence as described before (1, 28). Cell preparations were treated with mouse monoclonal antibody to β-galactosidase as the primary antibody and sheep anti-mouse IgG conjugated to FITC as the second antibody. Microscopic examination of these cell preparations indicated the presence of an intensely fluorescent region in DTY20(pMS116) cells which was not present in DTY20(YEp13) cells (Fig. 7). Visualization of the same cell preparations stained with DAPI allowed the assignment of this fluorescent region as the yeast cell nucleus. The location or relative intensity of the fluorescence in

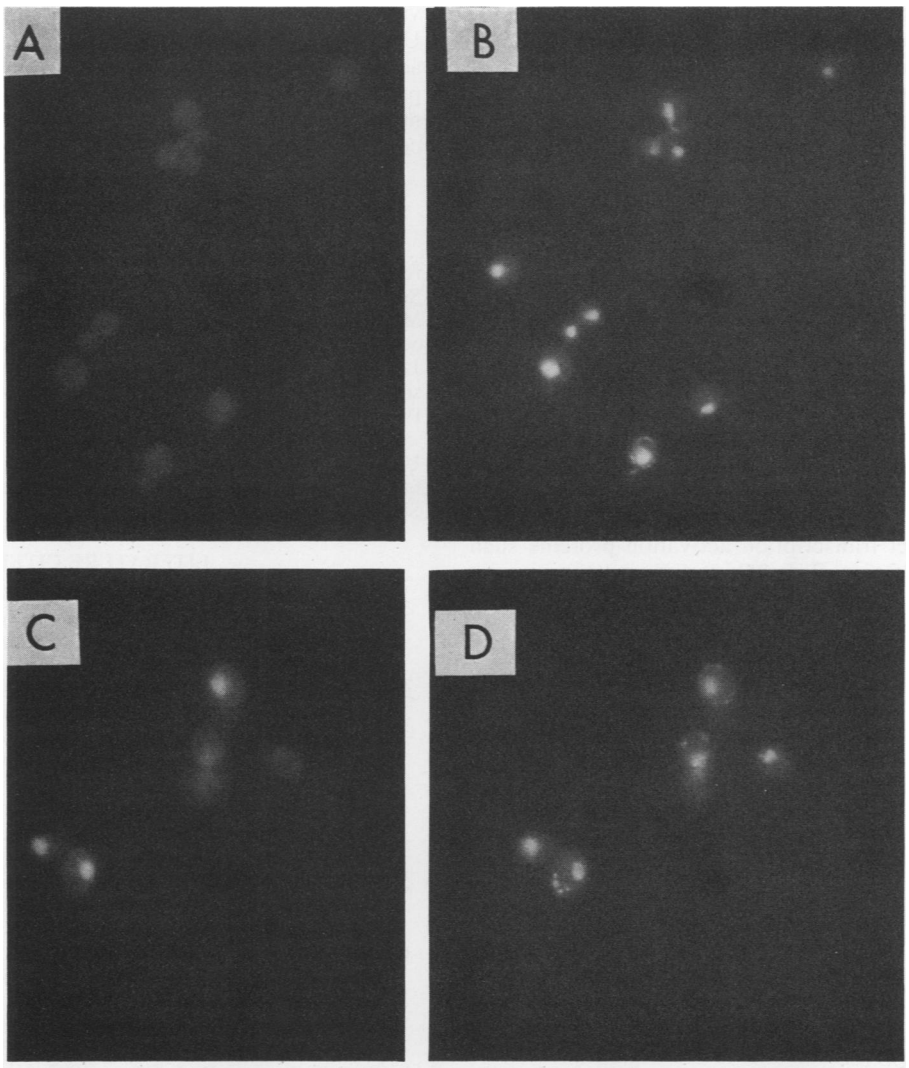

FIG. 7. *ACE1-lacZ* fusion protein accumulates in yeast cell nuclei. DTY20 cells harboring plasmid YEp13 (A and B) or the *ACE1-lacZ* fusion plasmid pMS116 (C and D) were treated with mouse monoclonal anti-β-galactosidase antibody and then sheep anti-mouse IgG antibody coupled to FITC (A and C). Each sample was also stained with DAPI, which stains the nucleus and mitochondria (B and D).

DTY20(pMS116) cells was not altered in cultures treated with copper sulfate under conditions known to induce *CUP1* gene transcription. Nuclear localization of the *ACE1*–β-galactosidase fusion protein was also observed in the wild-type strain H9 harboring pMS116 (data not shown). Consistent with a previous report for the intracellular location of authentic yeast metallothionein (44), a *CUP1*–β-galactosidase fusion protein expressed in strain DTY22 was localized throughout the yeast cell cytoplasm (M. Szczypka and D. Thiele, unpublished observations). The accumulation of the *ACE1*–β-galactosidase fusion protein in the yeast cell nucleus strongly suggests that the authentic *ACE1* gene product is a nuclear protein.

DISCUSSION

The yeast *ACE1* gene is an important genetic element required for copper-inducible transcription of the *CUP1* gene (39). Strains that carry the *ace1-1* or *ace1-Δ1* allele fail to induce transcription from either chromosomal or episomal copies of the *CUP1* promoter (39). The action of *ACE1* on *CUP1* copper-inducible transcription requires the presence of the *cis*-acting UAS_{CUP1} element, indicating that *ACE1* interacts directly or indirectly with *CUP1* transcriptional control sequences (M. Szczypka and D. Thiele, unpublished observations). Consistent with this observation is the finding that cells which bear the nonfunctional *ace1-Δ1* allele fail to demonstrate a copper-inducible interaction between one or more cellular factors and UAS_{CUP1} DNA sequences (Huibregtse et al., in press).

Here we present an analysis of the *ACE1* gene sequence and transcription unit. A single long ORF for a polypeptide of 225 amino acids (ORF-225) was present on the *ACE1* DNA sense strand. Evidence for the expression of the *ACE1* ORF-225 in yeast cells stems from several experimental observations. First, a 4-bp insertion at the *Bcl*I restriction enzyme site (plasmid pMS114) rendered the *ACE1* clone unable to complement the *ace1-1* mutation. This insertion would generate a frameshift mutation in ORF-225 so that this reading frame terminated 14 codons downstream from the *Bcl*I site. Second, we have shown that the carboxy-terminal half of ORF-225 was encoded within a 1-kb *Bgl*II DNA

restriction fragment that is essential (39) but not sufficient for *ACE1* gene function. Third, fusion of 95% of ORF-225 in frame to the *E. coli lacZ* gene resulted in expression of β-galactosidase activity in yeast cells. Furthermore, the expression of intact ORF-225 and deletion derivatives as fusion proteins in *E. coli* gives rise to fusion proteins of the size predicted from the *ACE1* gene sequence (M. Szczypka and D. Thiele, unpublished observations).

The analysis of *ACE1*-specific RNA suggests that steady-state levels of *ACE1* mRNA are not regulated by environmental copper levels. Consistent with this result, we observed no sequences in the *ACE1* gene upstream region bearing significant homology to UAS_{CUP1} (40). Similarly, the expression of an *ACE1*–β-galactosidase fusion protein, and presumably the authentic *ACE1* protein, is not regulated significantly by copper. These results are consistent with the possibility that the stability or activity of the *ACE1* gene product is modified posttranslationally. One possibility is that the *ACE1* protein, by virtue of cysteine or histidine residues, must bind copper in order to activate *CUP1* gene transcription. Direct transcription activation proteins such as *GAL4* (22), *SWI5* (34), Sp1 (25), and *Xenopus* transcription factor IIIA (18) require zinc as a cofactor for sequence-specific DNA binding. Although these data provide no evidence for a DNA-binding function for the *ACE1* protein, our observation that cells lacking a functional *ACE1* allele failed to demonstrate copper-inducible UAS_{CUP1} binding activity is consistent with this possibility (Huibregtse et al., in press).

The presence of 12 cysteine residues in the amino-terminal 105 amino acids of the putative *ACE1* gene product suggests a potential interaction of the *ACE1* protein with metals. Although the cysteine-rich region of ORF-225 bears no strong resemblance to the zinc-binding finger protein motif (2, 3, 22, 23, 25, 34), the occurrence of cysteine residues in the arrangement Cys-X-Cys and Cys-X-X-Cys corresponds to many of the metal-liganding regions in metallothionein proteins (26, 43). Yeast metallothionein has the ability to bind 8 mol of copper or 4 mol of zinc per mol of protein (43); however, the ability of the *ACE1* gene product to bind metals has not been reported. Another possible function of the cysteine residues in the putative *ACE1* gene product may be in the formation of intra- or interstrand disulfide linkages.

The data reported here support the possibility that *ACE1* may function as a direct activator of *CUP1* gene transcription. We observed that the protein expressed in yeast cells from the *ACE1-lacZ* gene fusion (pMS116) was concentrated in the cell nucleus. Similar fusions for known direct transcription activators in yeast cells, such as *HAP2* (35) and *GAL4* (38), are also directed to the yeast cell nucleus. The precise amino acid sequences necessary for transport of these and other (12) proteins to cell nuclei appear to have no strict homology. Although we have not determined the intracellular location of the *ACE1*–β-galactosidase fusion protein expressed from a single-copy gene, this result strongly suggests that the authentic *ACE1* protein is also concentrated in the yeast cell nucleus. We observed no difference in location of the fusion protein between recipient cells with an *ACE1* or *ace1-1* allele or between cells exposed or not exposed to exogenous copper, a condition known to activate *CUP1* gene transcription in an *ACE1*-dependent manner (39). Consistent with a nuclear site of action, we previously demonstrated that a functional *ACE1* allele is essential to detect the copper-inducible binding of one or more cellular factors to UAS_{CUP1} chromosomal DNA sequences (Huibregtse et al., in press). Together, these observations suggest that the *ACE1* protein may bind directly to UAS_{CUP1} or that the *ACE1* protein, acting in the nucleus, activates the synthesis or activity of a direct DNA-binding protein. In either case, the *ACE1* gene appears to encode a nuclear protein which is activated posttranslationally in a copper-dependent fashion.

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