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ACE2, an Activator of Yeast Metallothionein Expression Which Is Homologous to *SWI5*

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Transcription of the *Saccharomyces cerevisiae* metallothionein gene *CUP1* is induced in response to high environmental levels of copper. Induction requires the *ACE1* gene product, which binds to specific sites in the promoter region of the *CUP1* gene. In this study, we found that deleting the entire coding sequence of the *ACE1* gene resulted in a decrease in basal-level transcription of *CUP1* to low but detectable levels and conferred a copper-sensitive phenotype to the cells. We have isolated a gene, designated *ACE2*, which when present on a high-copy-number plasmid suppresses the copper-sensitive phenotype of an *ace1*-deletion strain. The presence of multiple copies of the *ACE2* gene enhanced expression of an unlinked *CUP1-lacZ* fusion integrated in the yeast genome and resulted in an increase in the steady-state levels of *CUP1* mRNA in an *ace1*-deletion background. A large deletion of the coding region of the genomic copy of *ACE2* resulted in a decrease in steady-state levels of *CUP1* mRNA, indicating that *ACE2* plays a role in regulating basal-level expression of *CUP1*. The *ACE2* open reading frame encodes a polypeptide of 770 amino acids, with putative zinc finger structures near the carboxyl terminus. This protein is 37% identical to the *SWI5* gene product, an activator of *HO* gene transcription in *S. cerevisiae*, suggesting that *ACE2* and *SWI5* may have functional similarities.

Regulation of gene expression in response to specific environmental changes has been extensively studied in a wide variety of eucaryotic cells. One model system for these studies is expression of the metallothionein (MT) genes, which encode small cysteine-rich metal-binding proteins that serve both to maintain intracellular levels of metals (17, 65) and to protect cells against heavy-metal toxicity (12, 29). Invertebrates and mammals contain multiple MT genes, and transcription is greatly increased in response to heavy metals such as cadmium, zinc, and copper (29, 33). Detailed analysis of the upstream region of the mouse MT-I gene (16, 19, 53) and the human MT-II gene (40) by transfection of promoter deletion and point mutations has led to the identification of metal regulatory elements which mediate the response to metals. Similar sequences have also been identified in the promoter regions of MT genes of sea urchin (33), *Drosophila melanogaster* (57), trout (67), and rat (3). These small metal regulatory elements are sufficient to confer metal regulation to truncated promoter fusions (19, 59). *trans*-acting factors which bind to the metal regulatory elements of the mouse and trout MT genes have been detected in DNA binding assays (37, 47, 54, 55).

Transcription of MT genes in higher organisms is considerably more complex than a response to the presence of heavy metals. It has been shown that transcription of the human MT genes can be induced by other effector molecules such as glucocorticoids (44), interferon (24), phorbol esters (4), protein kinase C, and cyclic AMP-dependent protein kinase A (36). These effects are mediated through the binding of proteins such as AP1 (4, 42) and AP2 (36, 45) to elements in the MT promoter. A potential binding site implicated in transcriptional induction by interferon has been identified in the promoter of the human *MTIIA* gene (24), and other factors, including Sp1, bind to the mouse MT-I promoter (8, 38). The fact that a multitude of protein factors interact with MT promoters of higher eucaryotes indicates that these genes have a very complex promoter structure and are subject to multiple regulatory controls (29).

The MT protein in the yeast *Saccharomyces cerevisiae* is encoded by the *CUP1* gene (13, 14, 23). Resistance to copper is conferred both by amplification of the *CUP1* locus and by induction of *CUP1* gene transcription in the presence of copper (for reviews, see references 12 and 29). Copper-inducible transcription of *CUP1* is mediated through upstream activation sequences (UAS_{CUP1}) identified by promoter mutagenesis (63) and a *trans*-acting factor, the ACE1 protein. Genetic studies first identified the mutant *ace1-1* allele, which renders cells hypersensitive to copper poisoning as the result of an inability to carry out copper-inducible transcription of the *CUP1* gene (62). The *ACE1* gene (also known as *CUP2* [11, 64]), was cloned by complementation and shown to encode a small cysteine-rich protein with a positively charged amino terminus and an acidic carboxyl-terminal domain (61). Several lines of evidence have demonstrated that ACE1 is a DNA-binding protein. A functional *ACE1* gene is required for copper-inducible binding of cellular factors to the UAS_{CUP1} sequences (35). ACE1 protein synthesized in vitro binds, in a copper-inducible fashion, to a region of the UAS in *CUP1* that was previously identified by in vivo footprinting (25). This region includes at least three specific binding sites, detected by in vitro footprinting using a TrpE-ACE1 fusion protein (22). It is now known that two *ACE1* molecules bind to the recognition site closest to the start site of transcription (34).

The presence of the *ACE1* gene on high-copy-number plasmids has been shown to result in an increase in basal-level transcription of *CUP1* (25, 35). It has also been shown that a mutant allele (*cup2*) of the *ACE1* gene results in a reduction in transcription of *CUP1* in the absence of exogenous copper, to below levels detectable by RNA blotting (64). These results suggest that the *ACE1* gene product is involved in maintenance of basal-level expression as well as in copper-inducible transcription of *CUP1*. Furthermore,

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TABLE 1. Yeast strains

Strain	Genotype	Source or reference
H9	*MATα his6 leu2-3,112 ura3-52 CUP1*$^{R-3}$	62
DTY22	*MATα his6 ura3-52 leu2-3,112 LEU2*:: YipCL *CUP1*$^{R-3}$	62
DTY59	*MATα his6 leu2-3,112 ura3-52 ace1-Δ225 CUP1*$^{R-3}$	This work
DTY60	*MATα his6 leu2-3,112 ura3-52 ace1-Δ225 LEU2*::YipCL *CUP1*$^{R-3}$	This work
DTY91	*MATα his6 leu2-3,112 ura3-52 ace2-Δ439::URA3 ace1-Δ225 CUP1*$^{R-3}$	This work
DTY93	*MATα his6 leu2-3,112 ura3-52 ace2-Δ439::URA3 CUP1*$^{R-3}$	This work

CUP1 itself has been demonstrated to play a negative autoregulatory role in its transcription (30), presumably by binding copper ions that might otherwise be available to ACE1 (66). No other *trans*-acting factors, apart from ACE1, involved in regulation of *CUP1* transcription have been identified. Here we show that deleting the entire coding sequence of the *ACE1* gene results in sensitivity to low copper levels as the result of a decrease in transcription of the *CUP1* gene to low but detectable levels. We have isolated a gene, designated *ACE2*, which acts as a specific high-copy-number suppressor of the copper-sensitive phenotype of an *ace1* deletion by increasing the basal levels of *CUP1* mRNA. The primary structure of the *ACE2* gene suggests that it encodes a DNA-binding protein. We present evidence that the *ACE2* gene may play a role in the activation of basal-level expression of *CUP1*.

MATERIALS AND METHODS

Strains, media, and enzyme assays. The yeast strains used in this study are shown in Table 1. Yeast strains were grown in standard rich (YPD) or synthetic complete (SC) medium lacking specific nutrients as described previously (21, 56). Induction with copper was carried out by adding copper sulfate to a final concentration of 50 to 500 μM to exponentially growing cells (optical density at 650 nm of 1 to 1.5) and incubating the cells at 30°C, 300 rpm for 45 min. Plasmids were constructed and maintained in *Escherichia coli* DH5αF', using standard techniques (43). β-Galactosidase assays were carried out as described previously (28).

Plasmids. The *ACE2* gene was isolated as a 7.7-kb insert from a DNA library constructed from *S. cerevisiae* DBY939 partially digested with *Sau*3AI and cloned in the *Bam*HI site of the high-copy-number vector YEp24 (15). Subclones were constructed by isolation of restriction fragments which were cloned into the *Bam*HI, *Sma*I, or *Sal*I site of YEp24 (see Fig. 2). YEp1 contains a 4.5-kb *Bam*HI fragment inserted into the *Bam*HI site of YEp24, YEp2 has a 2.2-kb *Bgl*II fragment inserted in the *Bam*HI site, and YEp3 contains a 2.3-kb *Hae*III fragment inserted into the *Sma*I site. YEpACE2r was constructed by isolating a fragment from the *Hpa*I site upstream from the *ACE2* gene to the *Sal*I site in the YEp24 sequences. This fragment was then used to replace the *Sma*I-*Sal*I fragment in YEp24. All inserts are in the same orientation relative to the plasmid flanking sequences.

Sequence analysis of *ACE2*. A 2.93-kb *Hpa*I-*Hae*III restriction fragment encompassing the *ACE2* gene was sequenced by cloning a series of overlapping internal restriction fragments into either M13mp18 and M13mp19 or Bluescript (Stratagene). The sequence of both strands was determined by using the Sequenase dideoxy-chain termination kit (U.S. Biochemical Corp.). Sequence analysis was carried out by using the programs of the University of Wisconsin Genetics Computer Group, and data library searches were done by the method of Pearson and Lipman (50).

RNA analyses. Total RNA was isolated from 1.5- to 5-ml cultures grown to log phase (optical density at 650 nm of 1 to 1.5) in SC medium lacking specific nutrients for plasmid maintenance and induced with copper sulfate where indicated (32). For Northern (RNA) blot analysis, 15 μg of total RNA was subjected to electrophoresis on a 1% formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated 1.3-kb *Eco*RV-*Bam*HI probe internal to the *ACE2* open reading frame. The size of the *ACE2*-specific mRNA was estimated by using RNA molecular weight markers from Bethesda Research Laboratories.

Primer extension analysis of *CUP1*-specific RNA was carried out by using 15 or 30 μg of total RNA per reaction, an oligonucleotide primer (5'-TTGATTGATTGTACAG-3', complementary to nucleotides +33 to +48 of the *CUP1* gene) 5' end labeled with [γ-^{32}P]ATP, and avian myeloblastosis virus reverse transcriptase as described previously (61). mRNA from the alcohol dehydrogenase gene (*ADH1*) was detected by primer extension reactions using a specific 17-mer oligonucleotide primer (5'-CGTAGAAGATAAC ACCT-3') complementary to nucleotides +24 to +40 (6). The start site of transcription of the *ACE2* gene was determined by using 25 μg of total RNA and the oligonucleotide primer 5'-TATACCACGGATCTACA-3', complementary to nucleotide positions +12 to +28 in Fig. 3. Labeled products were subjected to electrophoresis on 8% polyacrylamide-urea sequencing gels and exposed to Kodak X-AR film at −70°C with intensifying screens. Extension products were quantitated from several independent experiments by using laser densitometry (Quick Scan; Helena Laboratories). When the *CUP1* signal was weak and required long exposure times for detection, the *ADH1* signal was measured from shorter exposures to ensure that both signals lay within the linear range.

Construction of chromosomal deletion alleles of the *ACE1* and *ACE2* genes. A deletion of the *ACE1* gene was constructed as follows (Fig. 1). The *ACE1* gene was isolated on a 3.2-kb *Eco*RI fragment from plasmid pRI-3 (62) and inserted into the *Eco*RI site of plasmid pUC18. A *Bam*HI linker was inserted at nucleotide position −21 with respect to the major transcription start, using a deletion generated with the exonuclease BAL 31 from the *Nco*I site 5' to the *ACE1* reading frame (61a). Digestion with *Bam*HI and *Bgl*II removed a 1.5-kb fragment containing the entire *ACE1* coding sequence and approximately 800 bp of 3'-flanking sequence. This was replaced with a 3.8-kb *Bam*HI-*Bgl*II cassette containing the *URA3* gene flanked by two direct repeats derived from the *E. coli hisG* gene (2). The resulting 5.5-kb fragment was isolated after digestion with *Eco*RI and *Ssp*I and used to transform *S. cerevisiae* H9 (62). Several URA$^+$ transformants were purified and grown on SC plates containing 5-fluoro-orotic acid (21) to select for cells having undergone recombination between the *hisG* repeats, resulting in loss of the *URA3* gene. The final strain, DTY59, has a deletion of the entire *ACE1* gene and is ura$^-$. The *CUP1-lacZ* fusion was introduced into DTY59 by integrating plasmid YIpCL (62) at the *LEU2* locus to generate strain DTY60. All chromosomal alterations were verified by Southern blotting (43).

To construct a partial deletion of the *ACE2* gene, a 2.2-kb

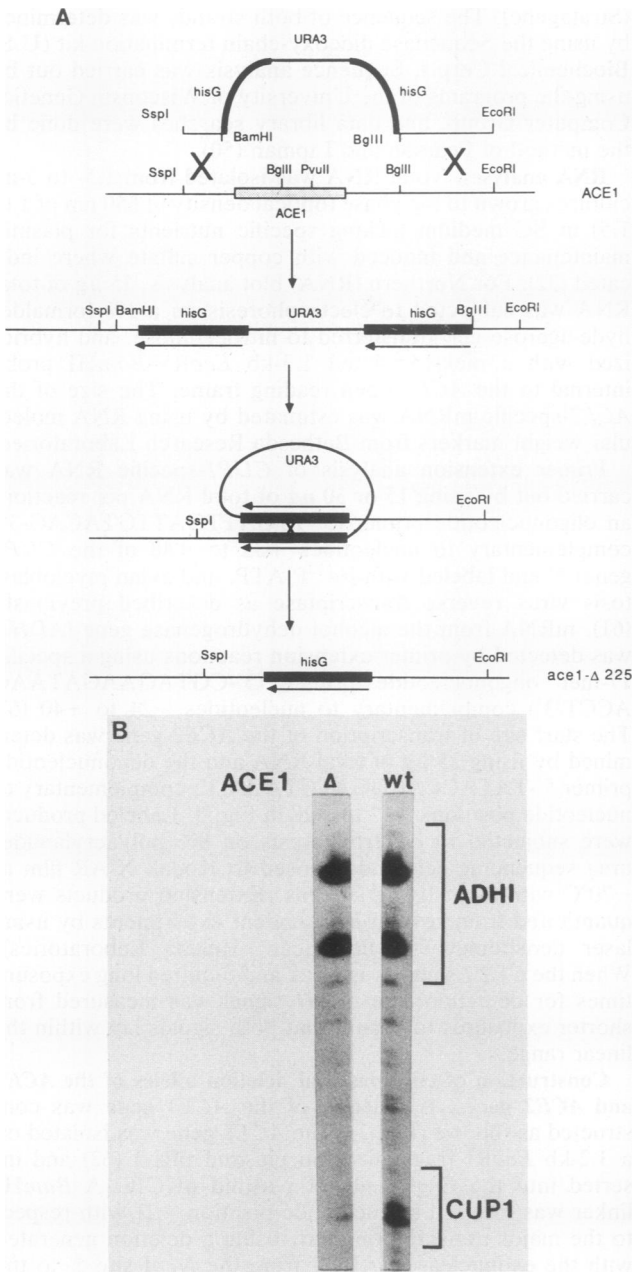


FIG. 1. Reduction of basal-level expression of *CUP1* upon deletion of the *ACE1* gene. (A) The entire coding sequence of the *ACE1* gene was deleted as shown schematically and described in Materials and Methods. The *ACE1* gene was first replaced with *URA3* cassette, flanked by direct repeats derived from the *hisG* gene. Recombination between the repeats results in the loss of the *URA3* marker, generating the deletion allele *ace1-Δ225*. (B) Total RNA was isolated from isogenic strains DTY60 (*ace1-Δ225*) and DTY22 (*ACE1*). *CUP1*- and *ADH1*-specific mRNAs (indicated with brackets) were detected by primer extension analysis using 30 μg of RNA. Lanes: Δ, *ace1*-deletion mutant; wt, *ACE1* wild type.

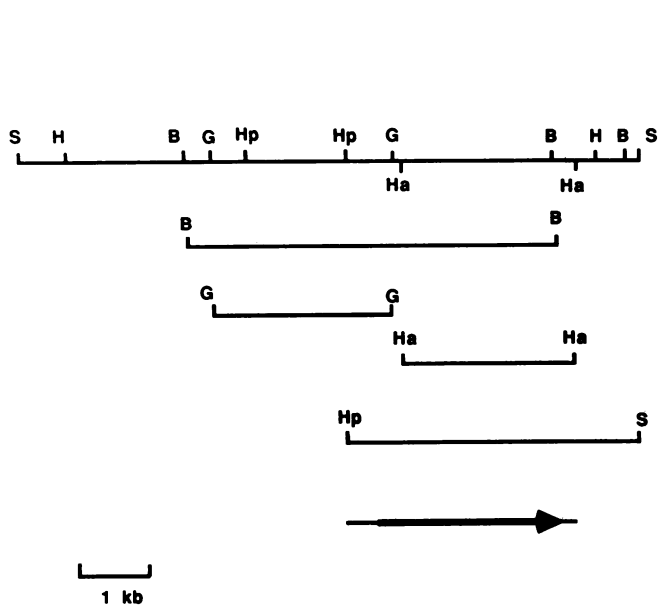
*Bgl*II-*Hae*III fragment of the *ACE2* gene was first cloned into compatible sites of plasmid pUC19. The resulting construct was cleaved with *Eco*RV and *Sma*I, which released a 1.3-kb fragment. A 1.2-kb *URA3* fragment isolated from YEp24 (with ends filled in with Klenow enzyme) was inserted into this recipient plasmid. This resulted in replacing 439 codons of the *ACE2* open reading frame with the *URA3* fragment. The modified *ACE2* fragment was liberated by digestion with *Eco*RI and *Hin*dIII in the pUC19 polylinker and transformed into yeast strains DTY59 and H9. Several independent URA^+ transformants were selected, and the chromosomal organization of this *ace2* allele (*ace2-Δ439*) was confirmed by Southern blotting (43).

Nucleotide sequence accession number. The sequence of the *ACE2* gene has been assigned GenBank accession number M55619.

RESULTS

Deletion of the *ACE1* gene lowers basal-level expression of *CUP1*. Copper-inducible transcription of the *CUP1* gene requires binding of the ACE1 protein to specific sites in the promoter region of *CUP1* (11, 22, 25, 35, 62). However, experimental evidence suggests that *ACE1* may also play a role in basal-level transcription of *CUP1* (25, 35, 62, 64). To clarify the potential role of *ACE1* and other factors in regulating basal-level transcription of *CUP1*, we constructed a deletion of the genomic copy of the *ACE1* gene (*ace1-Δ225*) (Fig. 1A). This deletion removes the entire coding sequence of *ACE1* and approximately 800 bp of 3′-flanking sequence. In a strain carrying three copies of the MT locus and the ace1-Δ225 allele, the cells are sensitive to low (<25 μM) levels of copper present in the medium. The isogenic wild-type parental strain tolerates copper sulfate levels up to approximately 1 mM. We compared the steady-state levels of *CUP1* mRNA in the isogenic *ACE1* and *ace1-Δ225* strains by primer extension analysis, which detected two major transcripts from the *CUP1* gene (Fig. 1B). These data demonstrate that deletion of the *ACE1* gene results in a 4- to 10-fold reduction in basal-level expression of *CUP1* but not of the control gene *ADH1* (encoding alcohol dehydrogenase). This sensitive primer extension assay detects a low basal expression level of *CUP1* mRNA that is not significantly increased by the addition of exogenous copper (data not shown). These results indicate that *ACE1*, as well as other cellular factors, contribute to *CUP1* expression in the absence of exogenous copper.

Isolation of a suppressor of the *ace1-Δ225* allele. To identify other regulatory factors involved in expression of *CUP1*, we searched for genes which, when present in multiple copies, suppress the requirement for *ACE1*. We used a yeast strain (DTY60) carrying three copies of the *CUP1* locus, a deletion of the entire coding sequence of the *ACE1* gene (*ace1-Δ225*), and a *CUP1-lacZ* fusion integrated at the *LEU2* locus (LEU2::YipCL [62]). Cells were transformed with a yeast genomic library inserted in the high-copy-number vector YEp24 (15), and copper-resistant transformants were selected by replica plating to SC-uracil medium containing 50 μM copper sulfate. From an initial screening of approximately 100,000 independent transformants, 66 copper-resistant isolates were identified. To identify factors involved in *trans* activation of *CUP1*, rather than those playing a role in copper transport or other copper homeostatic mechanisms, expression of a *CUP1-lacZ* fusion integrated at the *LEU2* locus was monitored by using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) on solid medium. Four blue colonies were identified, and the one with the most intense color was chosen for further study. Plasmid rescue and retransformation experiments demonstrated that the copper resistance phenotype and activation of the chromosomal *CUP1-lacZ* fusion were plasmid borne.



Plasmid	Cu^r	X-gal	β-gal -	β-gal +
Yep24	-	-	0	0
pGB1-1	++	++	23.9	20.3
YEp1	+	+	nd	nd
YEp2	-	-	nd	nd
YEp3	-	-	nd	nd
YEpACE2r	++	++	20.3	14.8

FIG. 2. Isolation of the *ACE2* gene. Plasmid pGB1-1 was isolated from a yeast DNA library in plasmid YEp24 transformed into *S. cerevisiae* DTY60 as a high-copy-number suppressor of an *ace1* deletion. pGB1-1 contains a 7.7-kb *Sau*3AI insert at the *Bam*HI site of the vector. Growth on SC medium containing 50 to 100 μM $CuSO_4$ is shown, (Cu^r column: −, no growth; +, growth on 75 μM $CuSO_4$; ++, growth on 100 μM $CuSO_4$), as is blue color on solid medium containing X-Gal (X-Gal column: −, white; +, light blue; ++, dark blue). The effect of fragments subcloned in plasmid YEp24 and retransformed into strain DTY60 is indicated. The levels of β-galactosidase (β-gal) were assayed from cells induced (+) or uninduced (−) with 100 μM $CuSO_4$. The bottom line shows the region of DNA sequenced, and the block arrow shows the position of the *ACE2* open reading frame and the direction of transcription. Abbreviations: nd, not determined; B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; Ha, *Hae*III (not all sites shown); Hp, *Hpa*I; S, *Sau*3AI (not all sites shown).

We assign the designation *ACE2* (for activator of *CUP1* expression 2) to the functional insert in this plasmid.

The original plasmid isolate (pGB1-1) has a DNA insert of approximately 7.7 kb in plasmid YEp24 and confers resistance to approximately 100 μM copper sulfate. To more precisely map the portion of DNA responsible for conferring resistance to copper and for activation of expression of the *CUP1-lacZ* fusion, fragments were subcloned into YEp24, retransformed into DTY60, and tested for copper resistance and β-galactosidase activity (Fig. 2). One plasmid (YEp1) contains a 4.5-kb *Bam*HI restriction fragment, which allows growth on medium containing up to 75 μM copper sulfate, with very low activation of *CUP1-lacZ* as observed on X-Gal plates. Subsequent sequence analysis showed that this clone is missing the last 23 amino acids of the open reading frame encoded by the full-length insert in pGB1-1. A 2.2-kb *Bgl*II fragment derived from the 5′ end of this clone (YEp2) does not confer resistance to copper. The *Hae*III restriction fragment cloned in plasmid YEp3 does not confer resistance to copper except when cloned in the opposite orientation in the same plasmid. This finding suggests that this fragment may contain most or all of the suppressor gene, but it is expressed only when a cryptic promoter is provided in one orientation by plasmid YEp24. The smallest fragment containing the *ace1*-deletion suppressor gene is a 3.7-kb *Hpa*I-*Sau*3AI fragment which confers resistance to copper levels of approximately 100 μM. β-Galactosidase levels determined for cells induced or uninduced with copper sulfate showed that the presence of the *ACE2* gene on this high-copy-number plasmid greatly activated expression of the *CUP1-lacZ* fusion in the *ace1-Δ225* mutant background (from 0 to approximately 20 β-galactosidase units), as in the original isolate pGB1-1, and that this induction was independent of exogenous copper (Fig. 2).

Primary structure of the *ACE2* gene. To begin to characterize the *ACE2* gene product, the sequence of the 2.93-kb *Hpa*I-*Hae*III fragment indicated in Fig. 2 was determined (Fig. 3). Subclone analysis shown in Fig. 2 suggests that this region contains the functional portion of the *ACE2* gene. This sequence contains only one large open reading frame, which has the capacity to encode a protein of 770 amino acids. A computer search of the GenBank and EMBL data bases for proteins resembling the predicted ACE2 polypeptide by using the algorithm of Pearson and Lipman (50) detected a very strong similarity (37% overall) to the SWI5 protein, a transcriptional activator of the HO endonuclease gene, involved in mating-type switching in *S. cerevisiae* (58). The strongest region of similarity is located near the carboxyl termini of the proteins, which also shows similarity to the zinc finger regions of the *Xenopus laevis* transcription factor TFIIIA and several other transcriptional activators. It has been shown in several instances that these structures, present in different numbers of repeats, represent DNA-binding motifs in which zinc coordination plays a major structural role (7, 41). Given this similarity, it is likely that *ACE2* encodes a DNA-binding protein. The extended similarity between *ACE2* and *SWI5* is presented in Fig. 7 will be discussed below.

The amino acid motif SPKK (or the extended motif SPXX, where X is any amino acid) is common in regulatory proteins, particularly surrounding DNA-binding domains such as zinc fingers (18, 60). It has been proposed that this sequence can itself bind to the minor groove of DNA (60). This motif is prevalent in the *ACE2* gene product, and it is

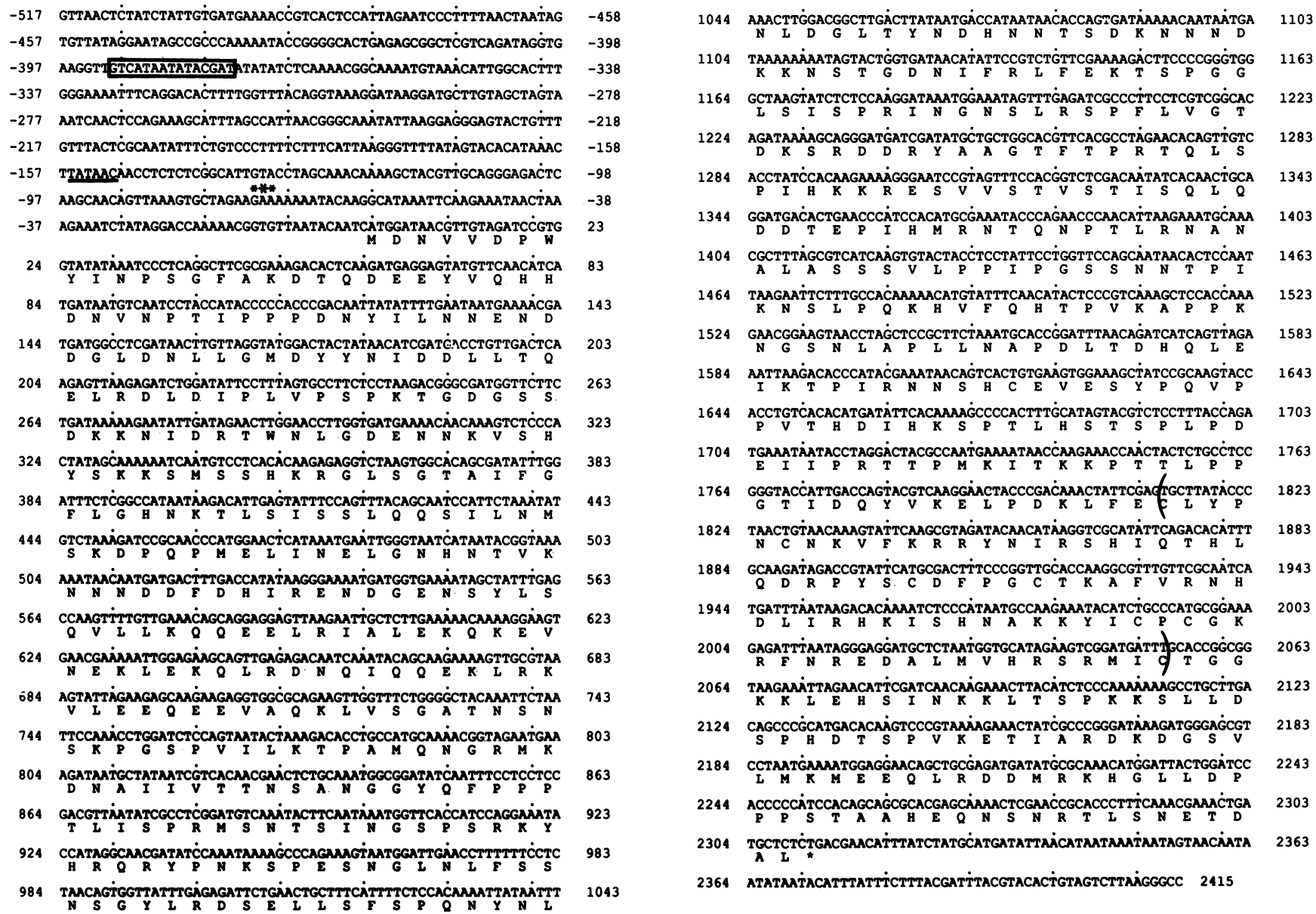


FIG. 3. Nucleotide sequence of the *ACE2* gene. The sequence of 2,932 nucleotides of the *ACE2* gene is shown, with the longest open reading frame indicated. The major and minor mRNA start sites, as determined by primer extension, are shown with large and small asterisks, respectively. A potential TATA box is underlined, and a possible binding site for the ABF1 protein is boxed. The zinc finger region is in parentheses.

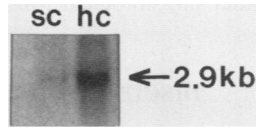


FIG. 4. Northern blot analysis of *ACE2*-specific RNA. Total RNA (15 μg per lane) was isolated from strain DTY60 (*ace1-Δ225*) transformed with either the high-copy-number plasmid YEp24 or the same vector with an *ACE2* insert (pGB1-1). The RNA was fractionated on a 1.5% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a 1.3-kb *Eco*RV-*Bam*HI fragment internal to the *ACE2* gene. The 2.9-kb *ACE2*-specific RNA species is indicated. Lanes: sc, single-copy *ACE2*; hc, high-copy-number *ACE2*.

interesting that one of these sequences (SPKK in ACE2) lying immediately 3′ to the zinc finger region of *ACE2* is conserved in *SWI5* (see Fig. 7A). However, the function of this sequence in the ACE2 protein remains to be investigated.

In the sequence upstream of the *ACE2* open reading frame, a putative TATA box is present between positions −156 and −151 (TATAAC). There is also a sequence between −391 and −377 (underlined in Fig. 3) with a strong similarity to the binding site for the ABF1 protein (9, 10). This element has been implicated in transcriptional control of several yeast genes, including those encoding subunits of the QH2-cytochrome *c* oxidoreductase (20), some ribosomal proteins (31), and the *DED1* gene (10), and therefore may represent a *cis*-acting transcriptional regulatory site in *ACE2*. However, the role of these sequence elements in expression of the *ACE2* gene has not yet been determined.

Characterization of *ACE2* mRNA. To characterize the *ACE2* mRNA species, total RNA was isolated from a yeast strain carrying a deletion of the *ACE1* gene (DTY60) transformed with either YEp24 or the *ACE2* high-copy-number plasmid pGB1-1. A 1.3-kb *Eco*RV-*Bam*HI probe internal to the *ACE2* open reading frame (see Fig. 6) detected a single RNA species of approximately 2.9 kb, consistent with the size of the proposed open reading frame (Fig. 4). The presence of the *ACE2* gene on a high-copy-number plasmid (pGB1-1) resulted in an increase in the level of the specific mRNA species detected. The levels of the wild-type *ACE2* RNA were approximately the same in isogenic *ACE1* wild-type and *ace1-Δ225* strains in several repeated experiments (data not shown), suggested that *ACE1* is not required for *ACE2* expression.

The major start site of transcription of *ACE2* was determined to be 73 bases upstream from the first ATG of the *ACE2* open reading frame by primer extension using an oligonucleotide complementary to the predicted transcribed strand. Two minor starts, one base upstream and downstream of the major start site, were also detected (data not shown). The location of the 5′ termini of the *ACE2* mRNA species is between the putative TATA box at positions −156 to −151 and the ATG which initiates the 770-codon open reading frame (Fig. 3).

Effect of *ACE2* on the expression of *CUP1*. To determine whether the *ACE2* gene functions as an activator of *CUP1* expression, the steady-state levels of *CUP1* mRNA from an *ace1*-deletion strain carrying either a single copy or multiple copies of *ACE2* were determined in a primer extension assay (Fig. 5). The two major *CUP1* primer extension products were detectable at low levels in the *ace1*-deletion strain and were increased 10- to 20-fold in the presence of a multicopy

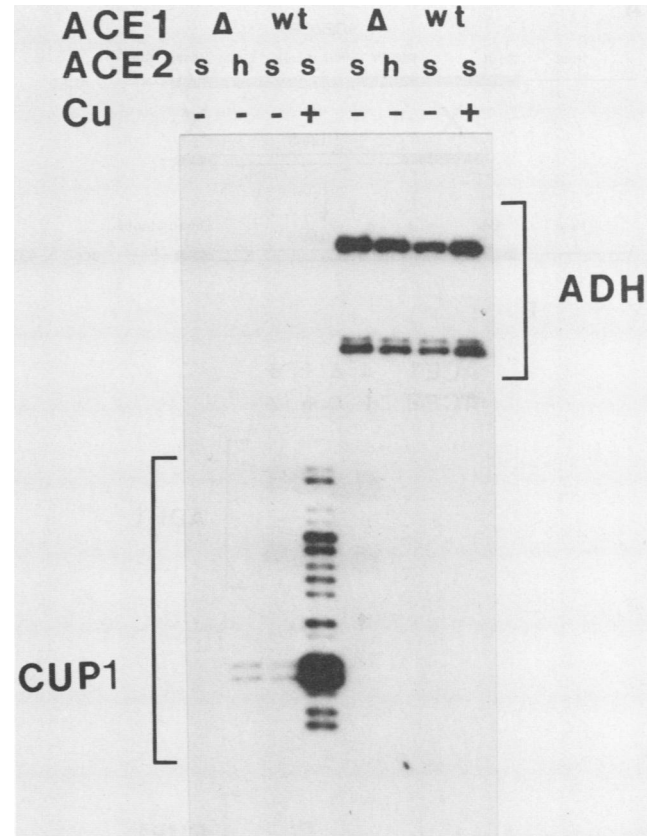


FIG. 5. Demonstration that multiple copies of the *ACE2* gene increase steady-state levels of *CUP1* mRNA. Total RNA was isolated from strain DTY60 (*ace1-Δ225*) transformed with either YEp24 or the high-copy-number *ACE2* plasmid pGB1-1 and from the isogenic strain DTY22 with a wild-type copy of *ACE1*. Samples induced (+) or uninduced (−) with 500 μM $CuSO_4$ (Cu) are indicated. Primer extension analysis of the *CUP1*-specific RNA was carried out by using 15 μg of RNA. Duplicate samples were used to measure levels of *ADHI* mRNA. The genotypes for the *ACE1* and *ACE2* loci are indicated (Δ, deletion; wt, wild type; s, single copy; h, high copy), and the *CUP1*- and *ADHI*-specific mRNA species are bracketed.

plasmid carrying the *ACE2* gene, as determined by laser densitometry from several independent experiments. This enhanced expression did not require the presence of exogenous copper in the medium, and the levels were not as high as in an *ACE1* wild-type background induced with copper sulfate. A similar increase in *CUP1* basal-level expression was seen when the *ACE2* gene was present in multiple copies in a wild-type *ACE1* background (data not shown). The levels of mRNA from an internal control gene, *ADHI*, were not increased in the presence of multiple copies of *ACE2* or during growth on copper, suggesting that the increase in the steady-state levels of *CUP1* mRNA is not part of a general increase in gene expression in response to multiple copies of the *ACE2* gene.

The *ACE2* gene affects basal-level expression of *CUP1*. Because *ACE2* was identified as a high-copy-number suppressor of an *ace1*-deletion allele, we tested whether *ACE2* plays any role in *CUP1* expression under physiological conditions. A partial deletion of the *ACE2* gene was constructed by replacing a DNA fragment encoding 439 amino acids from the C-terminal portion of the *ACE2* open reading

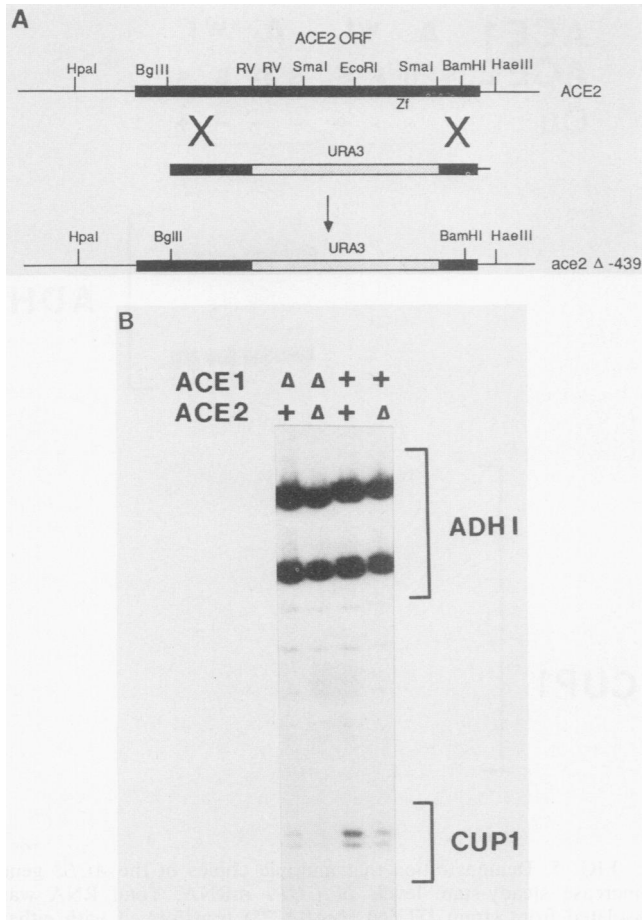


FIG. 6. Demonstration that a large deletion of the chromosomal *ACE2* open reading frame results in a decrease in steady-state levels of *CUP1* mRNA. (A) The *ACE2* gene was disrupted by replacing 439 amino acids of *ACE2* between the *Eco*RV and *Sma*I sites with the *URA3* gene. The modified *ACE2* fragment was used to replace the genomic copy of *ACE2* in isogenic strains carrying either a wild-type or deleted copy of the *ACE1* gene (H9 and DTY59). Abbreviations: RV, *Eco*RV; Zf, zinc finger region; ACE2 ORF, *ACE2* open reading frame. (B) Total RNA was isolated from yeast strains with the indicated genotypes, which were otherwise isogenic (Δ, deletion; +, wild type). The *CUP1*- and *ADH1*-specific mRNA species were detected by primer extension, using 30 μg of RNA. Both primers were used simultaneously, and the mRNA species are indicated with brackets. The *ace2*-deletion allele used here is *ace2-Δ439*, and the *ace1*-deletion allele is *ace1-Δ225*.

frame, including the potential zinc finger region, with the *URA3* gene (Fig. 6A). This altered *ACE2* allele was then used to replace the genomic copy of the *ACE2* gene in isogenic strains, carrying either a wild-type or deleted copy of the *ACE1* gene. These disruptions did not result in lethality in haploid yeast strains. The effect on steady-state levels of *CUP1* mRNA in these strains was monitored by primer extension (Fig. 6B). Deleting the carboxyl-terminal 439 amino acids of the *ACE2* open reading frame resulted in a fourfold decrease in the steady-state levels of *CUP1* mRNA in an *ace1*-deletion background and a twofold decrease in an *ACE1* wild-type strain, as determined by laser densitometry, but did not affect transcription of the *ADH1* gene. These results indicate that the *ACE2* gene plays a role in *CUP1* gene expression in the absence of exogenous copper, but the exact physiological function remains to be determined.

DISCUSSION

Metal-inducible transcription of the *CUP1* gene in *S. cerevisiae* requires the product of the *ACE1* gene (61, 62, 64). In the presence of copper, the ACE1 protein binds to at least three specific recognition sites in the *CUP1* promoter, resulting in a 10- to 50-fold increase in transcription. Overexpression of the *ACE1* gene can result in an increase in basal-level transcription of the *CUP1* gene (25, 35), and some *ACE1* mutant alleles (*cup2* [64]) reduce basal-level transcription of *CUP1* below levels detectable by Northern blot analysis. We have shown that deleting the entire coding sequence of the *ACE1* gene results in a reduction in basal-level transcription of *CUP1*, but RNA levels are still detectable with use of a sensitive primer extension analysis. We suggest that in the absence of exogenous copper, the ACE1 protein scavenges free copper ions in the cell, allowing binding to the *CUP1* promoter and activation of transcription. However, in the absence of the *ACE1* gene, *CUP1* is still transcribed, suggesting that other factors are also involved in basal-level expression. We have used the copper-sensitive phenotype of an *ace1* deletion to select for such factors. Here we describe the identification of a gene, *ACE2*, which when present in multiple copies in an *ace1*-deletion strain allows growth on a medium containing significant levels of exogenous copper sulfate. Subclone analysis has delineated a 3.7-kb fragment which confers resistance to 100 μM copper. Sequence analysis reveals only one continuous open reading frame, with the capacity to encode a protein of 770 amino acids.

When the *ACE2* gene is introduced into an *ACE1*-deletion strain on a high-copy-number plasmid, expression of a *CUP1-lacZ* fusion is increased in a copper-independent fashion, and steady-state levels of *CUP1* mRNA, but not of a control gene, *ADH1*, are increased 10- to 20-fold. This is accompanied by a corresponding increase in the amount of *ACE2*-specific mRNA. Deleting a portion of a genomic copy of *ACE2* results in a fourfold reduction in steady-state *CUP1* RNA levels in an *ace1*-deletion background. This reduction is less dramatic (approximately twofold) when the *ACE1* gene is present, suggesting that the *ACE1* protein masks the effect of *ACE2*. It is interesting that similar basal-level effects originally identified in expression of mammalian MTs are now known to be the result of a response to phorbol esters through the action of the AP1 protein (4, 42). It is possible that the *ACE2* product is involved in the regulation of *CUP1* expression in response to yet unidentified physiological or environmental stimuli.

Sequence analysis of the *ACE2* gene suggests that it encodes a DNA-binding protein. There is a region near the carboxyl terminus of the proposed *ACE2* gene product with strong similarity to the zinc finger regions of the *X. laevis* TFIIIA transcription factor (26), the brla protein of *Aspergillus nidulans* (1), the mammalian Sp1 protein (39), the *SWI5* gene product of *S. cerevisiae* (58; Fig. 7), and several other related proteins, as detected using the algorithim of Pearson and Lipman (50). It was originally proposed that these structures, which contain several copies of the consensus Cys_2-X_{12}-His_2, use the invariant cysteine and histidine residues to bind zinc ions. Several proteins containing zinc finger motifs have been shown to bind to DNA, and it has been demonstrated that the zinc finger region is required for DNA-protein interactions (7). The *ACE2* open reading frame

A

```
ACE2   1 MDNVVDPWYINPS..GFAKDTQDEEYVQHHDNVNPTIPPPDNYILNNE.. 46
SWI5   1 MD.TSNSWFDASKVQSLNFDLQTNSY.....YSNARGSDPSSYAIEGEYK 44

      47 ..NDDGLDNLLGMDYYNIDDLLTQELRDLDIPLVPSPKTGDGSSDKKNID 94
      45 TLATDDLGNILNLNYGETNEVIMNEINDLNLPLGPL......SDEKSVKV 88

      95 RTWNLGDENNKVSHYSKKSMSSHKRGLSGTAIFGFLGHNKTLSISSLQQS 144
      89 STFSELIGNDWQSMNFDLENNSREVTLNATSLLNENRLNQDSGMTVYQKT 138

     145 ILNMSKDPQPMELINELGNHNTVKNNNDDFDHIRENDGENSYLSQVLLKQ 194
     139 MSDKPHDEKKISMADNLLSTINKSEINKGFDR.........NLGELLLQQ 179

     195 QEELRIALEKQKEVNEKLEKQLRDNQIQQEKLRKVLEEQEEVAQKLVSGA 244
     180 QQELREQLRAQQEANKKLELELKQTQYKQQQLQATLENSDGPQ....... 222

     245 TNSNSKPGSPVILKTPAMQNGRMKDNAIIVTTNSANGGYQFPPPTLISPR 294
     223 .............FLSPKRKISPASENVEDVYANSL.........SPMISPP 252

     295 MSNTSINGSPSRKYHRQRYPNKSPESNGLNLFSSNSGYLRDSELLSFSPQ 344
     253 MSNTSFTGSPSRRNNRQKYCLQRKNSSG.............TVGPLCFQ 288

     345 NYNLNLDGLTYNDHNNTSDKNNNDKKNSTGDNIFRLFEKTS.PGGLSISP 393
     289 ELNEGFNDSLISPKKIRS..NPNENLSSKTKFITPFTPKSRVSSATSNSA 336

     394 RINGNSLRSPFLVGTDKSRDDRYAAGTFTPRTQLSPIHKKRESVVSTVST 443
     337 NITPNNLRLDFKINVED.QESEYSEKPLGLGIEL..LGKPGPSPTKSVSL 383

     444 ISQLQDDTEPIHMRNTQNPTLRNANALASSSVLPPIPGSSNNTPIKN..S 491
     384 KS.........................ASVDIMPTIPGSVNNTPSVNKVS 408

     492 LPQKHVFQHTP.....................VKAPPKNGSNLAPLLN 518
     409 LSSSYIDQYTPRGKQLHFSSISENALGINAATPHLKPPSQQARHREGVFN 458

     519 APDLTDHQLEIKTPIRNNSHCEVESYPQVPPVTHDIHKSPTLHST..... 563
     459 DLDPNVLTKNTDNEGDDNEENEPESRFVISETPSPVLKSQSKYEGRSPQF 508

     564 SPLPDEIIPRTT..PMKITKKPTTLPPGTIDQYVKELPDKLFECLYPNCN 611
     509 GTHIKEINTYTTNSPSKITRKLTTLPRGSIDKYVKEMPDKTFECLFPGCT 558

     612 KVFKRRYNIRSHIQTHLQDRPYSCDFPGCTKAFVRNHDLIRHKISHNAKK 661
     559 KTFKRRYNIRSHIQTHLEDRPYSCDHPGCDKAFVRNHDLIRHKKSHQEKA 608

     662 YICPCGKKFNREDALMVHRSRMICTGGKKLEHSINKKLTSPKKSLLDSPH 711
     609 YACPCGKKFNREDALVVHRSRMICSGGKKYENVVIKR..SPRKRGRPRKD 656

     712 DT.....SPVKETIARDKDGSVLMKMEEQLRD....DMRKHGLLDPPPST 752
     657 GTSSVSSSPIKENINKDHNGQLMFKLEDQLRRERSYDGNGTGIMVSPMKT 706

     753 AAHEQNSNRTLSNETDAL 770
     707 NQR.............. 709
```

B

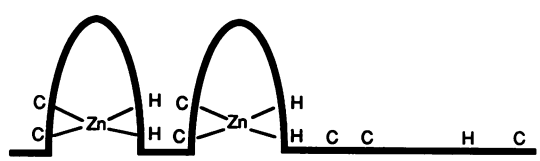


FIG. 7. Comparison of the ACE2 and SWI5 gene products. (A) The amino acid sequences of the *ACE2* and *SWI5* genes were linearly aligned by using programs from the University of Wisconsin Genetics Computer Group. Bars linking amino acids indicate identity; a comparison value of 0.5 or greater is indicated by a colon (:), and a value of 0.1 or greater is indicated by a period, based on evolutionary relationships of amino acids as normalized by Gribskov and Burgess (27). The region indicated with bold underlining has the potential to form zinc finger structures, and the residues conserved with the TFIIIA zinc finger consensus are indicated with stars. The underlined SPKK motif is discussed in the text. (B) The potential structure of the underlined zinc finger region in panel A is shown schematically. It is not known whether the CCHC sequence has the capability to form a finger.

contains a sequence possessing the invariant cysteine and histidine residues and several other conserved amino acids observed in the TFIIIA consensus (7; Fig. 7), with the capability to form two Cys_2-X_{12}-His_2-like zinc fingers. Although it is likely that the *ACE2* gene encodes a sequence-specific DNA-binding protein, we have been unable to detect a specific complex with a *CUP1* promoter fragment by using extracts from cells containing *ACE2* in high copy number by gel retardation experiments (61a). Whether ACE2 protein binds to *CUP1* under unknown conditions or represents an indirect *CUP1* regulatory factor remains to be determined.

Immediately following the two putative zinc fingers of *ACE2*, there is a third region which resembles the zinc fingers but is missing the last histidine residue. Similar sequences are seen in the zinc finger domains of the SWI5 protein of *S. cerevisiae* (58), the human MBP1 (5) and Evi-1 (46) proteins, the X-fin protein of *X. laevis* (52), and the *Drosohila* su(Hw) and Krüppel proteins (49, 51). These fingers match a Cys_2-X_{12}-His-Cys (called CCHC) consensus, and contain many of the conserved amino acids such as phenylalanine and leucine found in the TFIIIA-like structures (7, 41). A CCHC motif found in retrovirus genes believed to encode single-stranded DNA-binding proteins does not show conservation at these sites and has different spacing between the cysteine and histidine residues and therefore may not be related (7). Although it has not been shown that these CCHC boxes are capable of binding zinc, their presence in a wide range of proteins, in conjunction with TFIIIA-like zinc fingers, suggests that they may play a role in binding to DNA. One possibility is that they are involved in specific sequence recognition. It has been demonstrated that the two zinc fingers from SWI5, in conjunction with this structure, are sufficient to direct specific DNA binding (48). It is clear that deleting the C-terminal domain of the *ACE2* open reading frame, including the zinc finger region, results in a decrease in steady-state levels of *CUP1* mRNA (Fig. 6). However the precise biological role that the consensus zinc finger region in *ACE2* plays in *CUP1* expression has not yet been determined.

Although both ACE2 and the yeast SWI5 proteins contain TFIIIA zinc finger sequences, the similarity between the two proteins greatly exceeds the conserved residues believed to be involved in DNA binding. In the zinc finger region shown in Fig. 7A, the two proteins are 83% identical and demonstrate 37% identity across the entire protein sequence. The extensive similarity between the two proteins suggests the existence of a family of DNA-binding proteins that play a role in the regulation of mating-type switching, *CUP1* transcription, and perhaps the transcription of other, as yet unidentified yeast genes.

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