ACE1 Transcription Factor Produced in *Escherichia coli* Binds Multiple Regions within Yeast Metallothionein Upstream Activation Sequences

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The ACE1 protein of *Saccharomyces cerevisiae* was expressed as a *trpE*-ACE1 fusion protein in *Escherichia coli* and shown to bind CUP1 upstream activation sequences at multiple regions in a copper-inducible manner. These binding sites contain within them the sequence 5'-TC(T)₄₋₆GCTG-3', which we propose constitutes an important part of the ACE1 consensus recognition sequence.

The CUP1 gene of the yeast *Saccharomyces cerevisiae* provides a convenient model system for studying metallothionein gene transcription (1). Copper-inducible transcription of CUP1 involves upstream activation sequences (UAS_{CUP1}) which were functionally identified by promoter mutagenesis (4, 10). Genetic studies have identified an unlinked gene, denoted ACE1, which is required for copper-inducible transcription of CUP1 (9). Cells bearing a mutation in the ACE1 gene are hypersensitive to copper poisoning because of a failure to activate CUP1 transcription in response to high environmental copper levels (9). The ACE1 gene, cloned by in vivo complementation, maps to the left arm of chromosome VII, 9 centimorgans centromere distal to *lys5* (9). This location is close to an independently identified yeast gene, CUP2, which was identified and cloned based on its function in copper detoxification (11). Because of their similar roles and map location, ACE1 and CUP2 may be identical genetic elements.

The precise mechanism by which ACE1 activates CUP1 transcription has been investigated by a variety of approaches. Fusion of the amino-terminal 95% of the ACE1 open reading frame to the *Escherichia coli* β -galactosidase gene resulted in the concentration of this fusion protein in the yeast nucleus, as determined by indirect immunofluorescence (8). Consistent with this observation, chromosomal footprinting experiments indicated that a functional ACE1 gene is required for the copper-inducible binding of one or more cellular factors to UAS_{CUP1} sequences (6). The introduction of multiple plasmid-borne copies of the ACE1 gene allowed the detection of three distinct copper-inducible UAS_{CUP1}-protein interactions (6). ACE1 protein synthesized in an in vitro eucaryotic translation system was shown to bind in a copper-inducible manner to a region of UAS_{CUP1} which partially overlaps one of the regions observed to be bound by chromosomal footprinting (4, 6). To determine whether the ACE1 protein alone binds to all of the sites previously found protected in chromosomal footprints, we carried out DNase I footprinting studies with a *trpE*-ACE1 fusion protein synthesized in *E. coli*.

A fusion was constructed between the 3- β -indoleacrylic acid-inducible *E. coli* *trpE* gene in plasmid pATH2 (2) and the entire ACE1 open reading frame minus the initiating methionine. The resulting plasmid, pDT506, expressed large quantities of an approximately 62-kilodalton protein corre-

sponding to the expected size for the *trpE*-ACE1 fusion protein (data not shown). Extracts from 3- β -indoleacrylic acid-induced cultures containing either pATH2 or pDT506 were prepared for in vitro DNase I footprinting studies (5). Because the *trpE*-ACE1 fusion protein is largely insoluble in *E. coli* (D. J. Thiele, unpublished data), we prepared urea-solubilized extracts by a modification of a method previously described (7). During renaturation after urea solubilization, extracts were dialyzed against buffer B (7) or buffer B containing 10 μ M CuSO₄. These extracts were used in DNase I footprinting experiments with ³²P-end-labeled CUP1 promoter fragments (Fig. 1). Figure 2 shows the results of DNase I footprinting experiments in which the entire CUP1 promoter region (fragment A) was used in binding reactions with *trpE* or *trpE*-ACE1 protein extracts. We observed no protection by the control *trpE* extracts under any conditions. The *trpE*-ACE1 extract, however, produced copper-dependent protection of UAS_{CUP1}. This was true whether the protein extract was renatured in the presence of 10 μ M CuSO₄ or whether 10 μ M CuSO₄ was included in the binding reaction. Under either condition, three distinct regions of UAS_{CUP1} were protected from DNase I cleavage, and this protection was abolished by inclusion of homologous competitor DNA in the binding reactions (data not shown). Two of the regions (Fig. 2, regions 1 and 3) were more clearly protected than the third (region 2) when complete UAS_{CUP1} was used. In agreement with the work of others (4), the copper(I)-binding reagent KCN abolished sequence-specific DNA binding (data not shown).

We compared the pattern of *trpE*-ACE1-mediated protection from DNase I cleavage with probe A to that observed on the noncoding strand of UAS_{CUP1} with probe B (Fig. 1). Figure 3A (lanes A) shows protection of the three regions of UAS_{CUP1} observed previously in Fig. 2. Figure 3B (lanes C) shows that three regions of protection from DNase I cleavage were also observed on the noncoding strand. The coordinates of these protected regions (summarized in Fig. 3C) overlap the regions of protection observed for the coding strand, although for each site protection of the two strands is offset. We consistently observed better protection of region 2 on the noncoding strand than on the coding strand. This observation and that of others (3) contrast with our chromosomal footprinting results: we did not observe good protection of region 2 on the noncoding strand (6). We also noted that DNase I cleavage sensitivity on the noncoding strand

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FIG. 1. Schematic diagram of DNA fragments used in the DNase I protection experiments. The *Bam*HI-*Eco*RI fragment of pUC:CRB contains the entire *CUP1* promoter (10). The three regions protected in chromosomal footprinting experiments are shown as regions 1, 2, and 3. The multiplication sign indicates the end that is 32 P labeled, and the arrow indicates the major start site for transcription of the *CUP1* gene.

downstream of region 1 was increased relative to the no-extract control and that the DNase I cleavage pattern was altered upstream of region 3 (Fig. 3B). The altered DNase I cleavage patterns observed here were also observed with a *trpE* extract and are assumed to be unrelated to *trpE-ACE1* activity (data not shown). Importantly, the in vitro footprinting results with the *trpE-ACE1* fusion protein are very similar to our previous observations of copper-inducible binding to UAS_{CUP1} , as assayed by chromosomal footprinting (6). Furthermore, these experiments were carried out in the absence of other eucaryotic proteins.

Chromosomal footprinting demonstrated copper-induced protein binding to one UAS_{CUP1} region in an *ACE1* wild-type strain that corresponded closely to region 1. The introduction into cells of multiple *ACE1* gene copies allowed the detection of three distinct copper-induced DNA-protein interactions within UAS_{CUP1} that closely resembled regions 1 through 3 (6). One interpretation of these observations is that region 1 represents a high-affinity binding site for *ACE1* protein. This interpretation would be consistent with the observation that an oligonucleotide sequence spanning region 1 was found to play a major role in UAS_{CUP1} -mediated copper-inducible transcription (4, 10). A slight preference of *ACE1* synthesized in vitro to bind to sequences corresponding to region 1 has been suggested by others (3). To determine if binding to one UAS_{CUP1} region is dependent on contacts at other regions, we generated three probes to separate UAS_{CUP1} -binding regions and carried out DNase I footprinting experiments. End-labeled fragments B, D, and E (Fig. 1) were used in these experiments with a *trpE-ACE1* extract renatured in the presence of 10 μ M $CuSO_4$. Region 1 in probe B was protected from DNase I cleavage to an extent comparable to that in probe A, which contains all three binding regions (Fig. 3A, lanes A and B). Additional protection observed in probe B at position -152 was not readily discernable in probe A. Probes D and E were protected at regions 2 plus 3 and 3 alone, respectively. These data clearly demonstrate that binding to individual UAS_{CUP1} regions by *ACE1* does not require contacts at adjacent regions. We consistently observed better protection of region 2 in probe D than in the full-length probe C. We also observed protection of site 3 upstream to approximately -194 when probe E was used, as compared with -200 in the intact probe (probe

FIG. 2. DNase I footprinting of UAS_{CUP1} with *trpE* or *trpE-ACE1*. DNase I footprinting was performed with *E. coli* extracts containing the *trpE* protein or the *trpE-ACE1* fusion protein that had been dialyzed in the absence (-) or presence (+) of 10 μ M $CuSO_4$. Copper sulfate was (+) or was not (-) added to the binding reaction before the addition of extract. DNA was labeled at the 3' end of fragment A, containing the entire UAS_{CUP1} region (Fig. 1). Three regions of protection from DNase I are indicated by the brackets. Nucleotide positions relative to the start site of transcription of the *CUP1* gene (+1) are indicated. Other regions of altered DNase I cleavage are discussed in the text.

C). It is possible that the differences observed between probes C and D or E are due to steric hindrance created by the *trpE* portion of the fusion or other unknown parameters; however, the relatively poor protection of region 2 in the intact probe (probe A or C) is consistent with our previous chromosomal footprinting results (6).

Figure 3C summarizes the regions on both strands of UAS_{CUP1} protected from DNase I cleavage by the copper-activated *trpE-ACE1* fusion protein. Within or overlapping each binding region lies the sequence 5'-TC(T)₄₋₆GCTG-3'; we propose that it represents a consensus sequence important for *ACE1* protein binding. This consensus sequence appears six times within the *CUP1* promoter (Fig. 3C, boxes). Four of the 5'-TC(T)₄₋₆GCTG-3' elements lie within DNA stretches clearly protected by copper-activated *trpE-ACE1*. The observation that regions protected from DNase I cleavage by *trpE-ACE1* do not completely cover these sequences in regions 1, 2, and 3 might only reflect limits on our assignments imposed by the lack of susceptibility of these regions to DNase I cleavage. We observed no comparable protection of the other two members of this consensus sequence centering at approximately -152 and -248; however, our results suggest that these sites may have a low

FIG. 3. DNase I footprinting of both strands of UAS_{CUP1}. The ³²P-labeled DNA fragments used are shown schematically in Fig. 1. (A) The fragments were ³²P end labeled at the 3' end of UAS_{CUP1}. (B) The label was at the 5' end of UAS_{CUP1}. Binding reactions were incubated either without (–) or with (+) an *E. coli* *trpE-ACE1* extract that had been dialyzed against 10 μM CuSO₄. The three regions protected from DNase I cleavage are indicated by brackets. Nucleotide positions relative to the start site of transcription of the *CUP1* gene (+1) are indicated. (C) UAS_{CUP1}-binding regions for *trpE-ACE1* protein. Brackets indicate regions on both strands protected from DNase I cleavage. Boxed regions indicate the proposed ACE1 protein-binding consensus elements.

affinity for *trpE-ACE1* protein. We observed *trpE-ACE1*-dependent alterations of the DNase I cleavage pattern near –245 on the coding strand (Fig. 3A, lanes A), although it was not a clear footprint. There was also clear protection at position –152 in the absence of regions 2 and 3 (Fig. 3A, lane B, +), which might be occluded by more upstream ACE1 binding in complete UAS_{CUP1}.

Two lines of evidence support our suggestion of the

sequence 5'-TC(T)₄₋₆GCTG-3' as an ACE1-binding site consensus sequence. First, transition mutations at 7 of 12 of the nucleotides within this sequence in binding site 1 were shown to cause a dramatic decrease in in vivo transcription activity (4). Second, both G residues in the sequence 5'-GCTG-3' within this consensus sequence were shown to be in close contact with ACE1 protein by a methylation interference assay (4; unpublished observations). Although other

flanking residues may contribute to transcription activation of *CUP1*, this sequence appears to play a major role in the recognition of multiple *ACE1*-binding sites in the *CUP1* promoter.

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LITERATURE CITED

1. Butt, T. R., and D. J. Ecker. 1987. Yeast metallothionein and applications in biotechnology. *Microbiol. Rev.* **51**:351-364.
2. Dieckmann, C. L., and A. Tzagoloff. 1989. Assembly of the mitochondrial membrane system. *J. Biol. Chem.* **260**:1513-1520.
3. Furst, P., and D. Hamer. 1989. Cooperative activation of a eukaryotic transcription factor: interaction between Cu(I) and yeast ACE1 protein. *Proc. Natl. Acad. Sci. USA* **86**:5267-5271.
4. Furst, P., S. Hu, R. Hackett, and D. Hamer. 1988. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. *Cell* **55**:705-717.
5. Galas, D., and A. Schmitz. 1978. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5**:3157-3170.
6. Huibregtse, J. M., D. R. Engelke, and D. J. Thiele. 1989. Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. *Proc. Natl. Acad. Sci. USA* **85**:65-69.
7. Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. J. Tjian. 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**:1079-1090.
8. Szczypka, M. S., and D. J. Thiele. 1989. A cysteine-rich nuclear protein activates yeast metallothionein gene transcription. *Mol. Cell. Biol.* **9**:421-429.
9. Thiele, D. J. 1987. *ACE1* regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Mol. Cell. Biol.* **8**:2745-2752.
10. Thiele, D. J., and D. H. Hamer. 1986. Tandemly duplicated upstream control sequences mediate copper-induced transcription of the *Saccharomyces cerevisiae* copper-metalllothionein gene. *Mol. Cell. Biol.* **6**:1158-1163.
11. Welch, J., S. Fogel, C. Buchman, and M. Karin. 1989. The *CUP2* gene product regulates the expression of the *CUP1* gene, coding for yeast metallothionein. *EMBO J.* **8**:255-260.