MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4778–4787
0270-7306/90/094778-10$02.00/0

Vol. 10, No. 9

A Single Amino Acid Change in CUP2 Alters Its Mode of DNA Binding

CARLA BUCHMAN,[1] PETRA SKROCH,[1] WENDY DIXON,[2]
THOMAS D. TULLIUS,[2] AND MICHAEL KARIN[1]

Department of Pharmacology, M-036, School of Medicine, University of California San Diego, La Jolla, California 92093,[1] and Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Received 20 March 1990/Accepted 31 May 1990

CUP2 is a copper-dependent transcriptional activator of the yeast *CUP1* metallothionein gene. In the presence of Cu^+ (and Ag^+) ions its DNA-binding domain is thought to fold as a cysteine-coordinated Cu cluster which recognizes the palindromic *CUP1* upstream activation sequence (UASc). Using mobility shift, methylation interference, and DNase I and hydroxyl radical footprinting assays, we examined the interaction of wild-type and variant CUP2 proteins produced in *Escherichia coli* with the UASc. Our results suggest that CUP2 has a complex Cu-coordinated DNA-binding domain containing different parts that function as DNA-binding elements recognizing distinct sequence motifs embedded within the UASc. A single-amino-acid substitution of cysteine 11 with a tyrosine results in decreased Cu binding, apparent inactivation of one of the DNA-binding elements, and a dramatic change in the recognition properties of CUP2. This variant protein interacts with only one part of the wild-type site and prefers to bind to a different half-site from the wild-type protein. Although the variant has about 10% of wild-type DNA-binding activity, it appears to be completely incapable of activating transcription.

In recent years a large number of metal-ion-dependent DNA-binding proteins have been discovered. Most of them use the zinc-binding finger motif for recognition of specific DNA sequences (21). The Zn finger, first detected in transcription factor IIIA (TFIIIA), is part of a DNA-binding domain in which a linear peptide segment is arranged around a Zn^{2+} ion, complexing the metal with two cysteines and two histidines (23, 26). Proteins that belong to this class contain several such fingers that participate in contacting DNA. From theoretical considerations, it was suggested that each of the TFIIIA fingers interacts with either 5.5 base pairs (bp) (29) or 3.5 bp (14) of DNA. However, systematic deletions of individual fingers from TFIIIA indicate that clusters of fingers interact with three different parts of the binding site (41). Although it was difficult to assign the exact specificity of each finger in these studies, it appears that individual fingers make different contributions to the overall specificity. Structural studies indicate that each Zn finger forms one α-helix that has a high density of basic and polar amino acid residues on one exposed face, which may contact the sugar-phosphate backbone and specific bases of the DNA target site (23). This helix may be analogous to the recognition helix of the helix-turn-helix motif (27). The exact mode of sequence recognition by Zn finger proteins remains to be elucidated.

The basis for target site specificity was explored by using steroid hormone receptors which are thought to contain another type of a Zn finger, in which the metal ion is proposed to be coordinated by four cysteine residues (3, 8). These proteins are supposed to contain two such Zn fingers, both of which are required for DNA binding as demonstrated by mutagenesis of individual cysteines (31). The various nuclear receptor-binding sites are palindromic and are recognized by dimeric receptor complexes (2, 37). Mutations that affect amino acids lying between the second pair of cysteines in the first Zn finger of the glucocorticoid and estrogen receptors generate receptors with altered recognition specificity (6, 24, 40). These residues may therefore participate in contacting the DNA. Although the second finger is essential for DNA binding, its role is unknown (3).

A third class of metal-dependent DNA-binding proteins is represented by the CUP2 (also known as ACE1) protein of *Saccharomyces cerevisiae* (4, 11, 35). CUP2 is the product of the *CUP2* (*ACE1*) gene, a regulatory gene controlling transcription of *CUP1*, the yeast metallothionein gene (36, 42). CUP2 is composed of 225 amino acids, of which 12 are cysteines. These cysteines are concentrated at the N-terminal half of the protein, which is also rich in basic amino acid residues. This part of the protein forms a metal-dependent DNA-binding domain recognizing sequences that form the Cu-dependent upstream activation sequences (UASc) of the *CUP1* gene (4, 11). Only two metal ions, Cu^+ and Ag^+, lead to formation of an active CUP2 protein. The same ions are also effective inducers of *CUP1* transcription. Metal ions like Zn^{2+} or Cd^{2+}, which do not induce *CUP1* (19), do not lead to the formation of active CUP2 (4, 11).

Upon binding of Cu^+, CUP2 was proposed to undergo a conformational change, forming a cysteine-coordinated Cu cluster that functions as the active DNA-binding domain (11). To shed more light on the possible structure and function of CUP2, we have produced wild-type CUP2 and variant proteins encoded by the *ace1-1* and *cup2* mutant alleles (36, 42) in *Escherichia coli* and studied their interaction with the UASc by a variety of biochemical procedures. Our findings suggest that the DNA-binding domain of CUP2 is complex, being composed of several elements that recognize distinct features of the UASc. A single-amino-acid change may affect the integrity of one of these elements, generating a protein with altered recognition specificity that interacts with a smaller target site. Binding of this protein to DNA does not lead to transcriptional activation.

* Corresponding author.

MATERIALS AND METHODS

Protein expression and purification. pBluescript subclones containing the *CUP2*, *cup2*, and *ace1-1* coding sequences (4) were digested with *Nco*I and *Bam*HI, gel purified, and ligated into an *Nco*I-*Bam*HI-digested pET-8c expression vector containing the highly specific and potent T7 RNA polymerase promoter (F. W. Studier, A. H. Rosenberg, and J. J. Dunn, Methods Enzymol., in press). *E. coli* BL21(DE3) was transformed with the resulting plasmids, pET · CUP2, pET · cup2, and pET · ace1. This strain carries the T7 RNA polymerase gene under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter. In addition, BL21(DE3) contains pLysS, coding for T7 lysozyme, which keeps the basal level of genes subcloned into the expression vector low. Cells were grown in M9ZB (Studier et al., in press) containing 20 μg of ampicillin per ml and 25 μg of chloramphenicol per ml and were induced with 0.4 mM IPTG and 0.25 mM $CuSO_4$ when cultures reached an optical density at 600 nm of 0.6 to 1.0. Cells were harvested after 1.5 h, suspended in 1/40 of the original volume of buffer E (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 5 mM $MgCl_2$, 15 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol [DTT]), frozen at −80°C, thawed at 37°C, sonicated, and centrifuged (32,000 × *g* for 20 min at 4°C). Protease inhibitors (2 μg of aprotinin per ml, 2 μg of pepstatin A per ml, 2 μg of leupeptin per ml, 5 μg of soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) were added to the lysate, which was then heat treated for 10 min at 70°C and spun as before. The supernatant was subjected to $(NH_4)_2SO_4$ precipitation, and the 20 to 40% $(NH_4)_2SO_4$ pellet was dialyzed against buffer F (25 mM HEPES-NaOH [pH 7.9], 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 100 mM NaCl) and run over a heparin-agarose column equilibrated with buffer F. Proteins were eluted with 0.1 M NaCl steps. All three of the CUP2 proteins eluted at 1.0 M NaCl. This procedure resulted in proteins that were at least 95% pure as judged by silver staining of sodium dodecyl sulfate-polyacrylamide gels (22). Protein concentrations were determined by the Bradford assay and their A_{210}. Copper content was determined by flame atomic absorption spectroscopy on a Hitachi 180-80 polarized Zeeman atomic absorption spectrophotometer.

Mobility shift, DNase I footprint, and methylation interference assays. For mobility shift and methylation interference assays, pUASc, containing the *CUP1* 5'-noncoding region from −145 to −105, was used. This plasmid was generated by cloning of the oligodeoxynucleotides

```
5'-TCGAAGGGAT GCGTCTTTTC CGCTGAACCG TTCCAGCAAA AAAGACTAG-3'
   3'-TCCCTA CGCAGAAAAG GCGACTTGGC AAGGTCGTTT TTTCTGATC AGCT-5'
```

into the *Sal*I site of pUC19. For hydroxyl radical and DNase I footprinting assays pHinf100 (4), which spans the *CUP1* region from −183 to −83, was used. Plasmids were digested with *Hin*dIII or *Eco*RI and filled with ^{32}P-deoxynucleoside triphosphates and avian myeloblastosis virus reverse transcriptase.

Mobility shift assays contained the indicated amounts of CUP2 proteins, 0.3 ng of probe, 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris hydrochloride (pH 7.9), 0.6 mM EDTA, and 0.6 mM DTT in a total volume of 10 μl. After a 30-min incubation on ice, reaction mixtures were loaded on 8% polyacrylamide gels (acrylamide/bisacrylamide, 40:1). Gels were preelectrophoresed for 1 h at 15 mA in 22.5 mM Tris-borate (pH 8.3)–0.5 mM EDTA. Electrophoresis was performed at room temperature. Gels were quantitated by using the Ambis radioanalytic imaging system.

For methylation interference assays, the insert of pUASc was partially methylated with dimethyl sulfate, precipitated twice in the presence of carrier DNA, and then used as the probe in mobility shift assays. Wet gels were exposed overnight at room temperature, and bound and free bands were excised, electroeluted onto DEAE membranes (NA45 paper; Schleicher & Schuell, Inc.), eluted with 1 M LiCl, 20% ethanol–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA, phenol-chloroform extracted, and ethanol precipitated in the presence of carrier DNA. Pellets were suspended in 100 μl of 10% (vol/vol) piperidine and incubated at 90°C for 30 min to cleave the DNA at the methylated guanine residues. Samples were lyophilized twice after being suspended in 50 μl of H_2O; they were then suspended in formamide containing 0.1% bromphenol blue and 0.1% xylene cyanol and electrophoresed on a 10% polyacrylamide sequencing gel.

DNase I footprinting reactions were performed as described previously (1). However, reactions contained 10 μg of bovine serum albumin and only 0.1 μg of poly(dI-dC).

Hydroxyl radical footprinting. Samples for hydroxyl radical footprinting experiments contained 23.5 ng of CUP2, 0.25 ng of single-end-labeled probe (50,000 cpm), 1 μg of bovine serum albumin, 1% polyvinyl alcohol, 20 ng of poly(dI-dC), 12.5 mM HEPES-NaOH (pH 8.0), 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM DTT in a total volume of 10 μl. The hydroxyl radical cleavage reaction was performed in a manner similar to that previously described (38). To allow protein-DNA binding to reach equilibrium, the solution was incubated on ice for 15 min. After warming to room temperature over 1 min, the cutting reaction was initiated by mixing, on the inner wall of the Eppendorf tube, 2 μl each of iron(II)-EDTA solution [8 mM iron(II), 16 mM EDTA], 0.024% H_2O_2, and 20 mM sodium ascorbate. The cleavage reagent was immediately allowed to mix with the DNA-protein solution. The final concentrations of the constituents of the cleavage reagent were 1 mM iron(II), 2 mM EDTA, 0.003% H_2O_2, and 20 mM sodium ascorbate. After 2 min at room temperature the reaction was stopped by addition of 5 μl of 0.1 M thiourea. A 4-μl volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to each sample. To separate the protein-bound DNA from the free DNA, samples were loaded on a 6% polyacrylamide gel (acrylamide/bisacrylamide, 80:1). Electrophoresis was performed at room temperature at 125 V for 3 h in a buffer consisting of 25 mM Tris-borate hydrochloride (pH 8.3) and 2.5 mM EDTA. Wet gels were autoradiographed for 1 h. The band corresponding to DNA bound to protein was excised, crushed, and eluted by soaking in buffer (500 mM ammonium acetate, 1 mM EDTA [pH 6.5]) at 37°C overnight. The samples were precipitated with ethanol twice, rinsed, dried, suspended in 3 μl of formamide-dye mixture (90% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), heated to 90°C for 5 min, and electrophoresed on a denaturing gel (8% polyacrylamide, 50% urea). The gel was dried, autoradiographed, and scanned with a Joyce-Loebl Chromoscan 3 densitometer.

In vitro transcription. In vitro transcription reactions were performed with HeLa whole-cell extracts prepared as previously described (25). Reactions contained 50 μg of extract in 50 μl. HeLa extract (25 μl) plus the indicated amounts of bacterially expressed CUP2 proteins in a HEPES buffer (20 mM HEPES-KOH [pH 7.9], 12 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) were mixed with 250

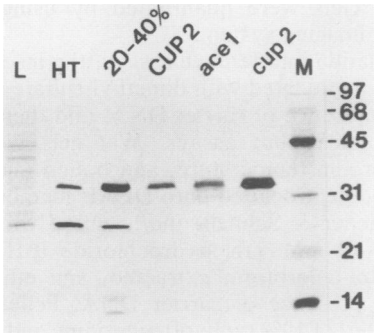

FIG. 1. Purification of wild-type CUP2 and the variant ace1 and cup2 proteins. Aliquots from each of the purification steps were analyzed on a sodium dodecyl sulfate–15% polyacrylamide gel stained with silver. Lanes: L, IPTG-induced soluble lysate of cells transformed with pET · CUP2; HT, heat-treated lysate; 20–40%, the 20 to 40% ammonium sulfate pellet; CUP2, protein after heparin-agarose chromatography; ace1 and cup2, purified variant proteins after heparin-agarose chromatography. Masses of protein gel markers (lane M) are indicated in kilodaltons.

ng of template DNA (200 ng of indicator, 50 ng of α-globin internal control) and incubated for 15 min on ice. After addition of 20 μl containing 1 mM each ribonucleotide and 5% polyvinyl alcohol, the samples were incubated at 30°C for 60 min. The reaction was stopped by addition of 90 μl of 1% sodium dodecyl sulfate–20 mM EDTA–200 mM NaCl–125 μg of yeast RNA per ml, and the products were subjected to phenol-chloroform extraction and ethanol precipitation. RNA transcripts were analyzed by primer extension with ^{32}P-labeled thymidine kinase (TK) and α-globin primers as described previously (7). Hybridizations were performed for 30 min at 65°C and 30 min at 55°C. The primer extension products were analyzed on a 10% denaturing polyacrylamide gel.

RESULTS

Expression of CUP2 proteins. To study the interaction of CUP2 with the *CUP1* promoter, the wild-type *CUP2* and the *cup2* and *ace1* mutant alleles were subcloned into pET-8c, a bacterial expression vector (Studier et al., in press). The *cup2* allele encodes a variant protein in which Gly-37 is replaced with a Glu residue, while the *ace1* mutation replaces Cys-11 with a Tyr residue (4). Both alleles were isolated by screening chemically induced mutants for ones defective in *CUP1* expression after metal induction (36, 42). Expression vectors were transformed into *E. coli*, and lysates were prepared from IPTG-induced cells (see Materials and Methods for details). $CuSO_4$ was added at the time of induction to produce Cu-activated binding proteins, as had been determined for trpE · CUP2 fusion proteins (4). Soluble material was heat treated, fractionated by $(NH_4)_2SO_4$ precipitation, and applied to a heparin-agarose column which was eluted with 0.1 M NaCl steps. This procedure resulted in proteins that were nearly homogeneous (Fig. 1). The CUP2 wild-type and variant proteins have apparent molecular masses of 33 kilodaltons, although their open reading frames have the potential to encode only a 24.4-kilodalton protein. This discrepancy reflects anomalous electrophoretic mobilities, which could be due to high local concentrations of basic residues as was described for other yeast transcription factors, including GCN4 (17) and HSTF (34). The metal content of the purified proteins was determined by flame atomic absorption spectroscopy and estimated to be six atoms of Cu per molecule of either CUP2 or cup2, and five atoms of Cu per molecule of ace1. Hence, replacement of Cys-11 with a Tyr residue apparently leads to a loss of one Cu-binding site.

DNA-binding activity of CUP2 proteins. To measure the DNA-binding activities of the CUP2 proteins, we used a modification of the gel electrophoresis DNA-binding assay (9, 13). Increasing amounts of protein were incubated with an end-labeled DNA fragment corresponding to the proximal UASc of the *CUP1* control region, from −105 to −148. Two discrete complexes were observed (Fig. 2A). The simplest interpretation of the data suggests that complex I is composed of a single CUP2 molecule and that complex II is composed of two protein molecules bound to the UASc (see below). After mixing of full-length and truncated CUP2 proteins and incubation with the UASc probe, we failed to find evidence that the protein binds DNA as a preformed dimer, even after denaturation-renaturation (data not shown). Therefore, it appears that complex I is generated by binding of a single protein molecule, whereas binding of a second molecule leads to formation of complex II. The mobilities of the complexes formed by the different proteins were essentially identical, although the bands corresponding to complex II formed by the variant proteins were broader than the wild-type complex II band.

Titration experiments (Fig. 2B) show that complex I forms first, with half-maximal binding observed at 5 nM CUP2. The two variant proteins are defective in binding as evidenced by half-maximal formation of complex I at 45 nM ace1 and 20 nM cup2. However, high concentrations of all proteins led to conversion of complex I into complex II (data not shown). Half-maximal formation of complex II was observed at 10 nM CUP2, 98 nM ace1, and 70 nM cup2.

Protein-DNA contacts. To determine the *CUP1* promoter regions protected by the different CUP2 proteins, we used DNase I (12) methylation interference (32), and hydroxyl radical (39) footprinting assays. End-labeled probes containing *CUP1* sequences from −183 to −83 were incubated with purified CUP2 proteins and then briefly digested with DNase I. CUP2 and cup2 protected the region from −146 to −104 on the top strand and −155 to −107 on the bottom strand. However, 200 times more cup2 was required for complete protection (Fig. 3). The boundaries of the CUP2 footprints agree with those of chromosomal footprinting experiments (18) and with the location of the proximal UASc as defined by mutational analysis of the *CUP1* promoter (11). By contrast, ace1 protected a much smaller region, corresponding to positions −130 to −110 on both strands. Ten times more ace1 than CUP2 was required for complete protection. Additional protections by the different proteins were seen over the distal UASc (data not shown).

Methylation interference analysis was used to investigate the guanine residues required for binding. Figure 4A shows that methylation of G-128, G-131, G-140, and G-142 on the top strand partially interfered with formation of CUP2-UASc complex I. Formation of this complex was also slightly inhibited by methylation of G-130 on the bottom strand (Fig. 4B). Formation of the slower-moving CUP2-UASc complex II was inhibited by methylation of G-109 and G-117 on the top strand (Fig. 4A) and G-116, G-119, and G-120 on the bottom strand (Fig. 4B), in addition to all of the G residues listed above whose methylation interfered with the formation of complex I. Inhibition of CUP2 binding by methylation of G-128, G-131, G-140, and G-142 was also observed by Furst et al. (11). However, these authors did not detect the

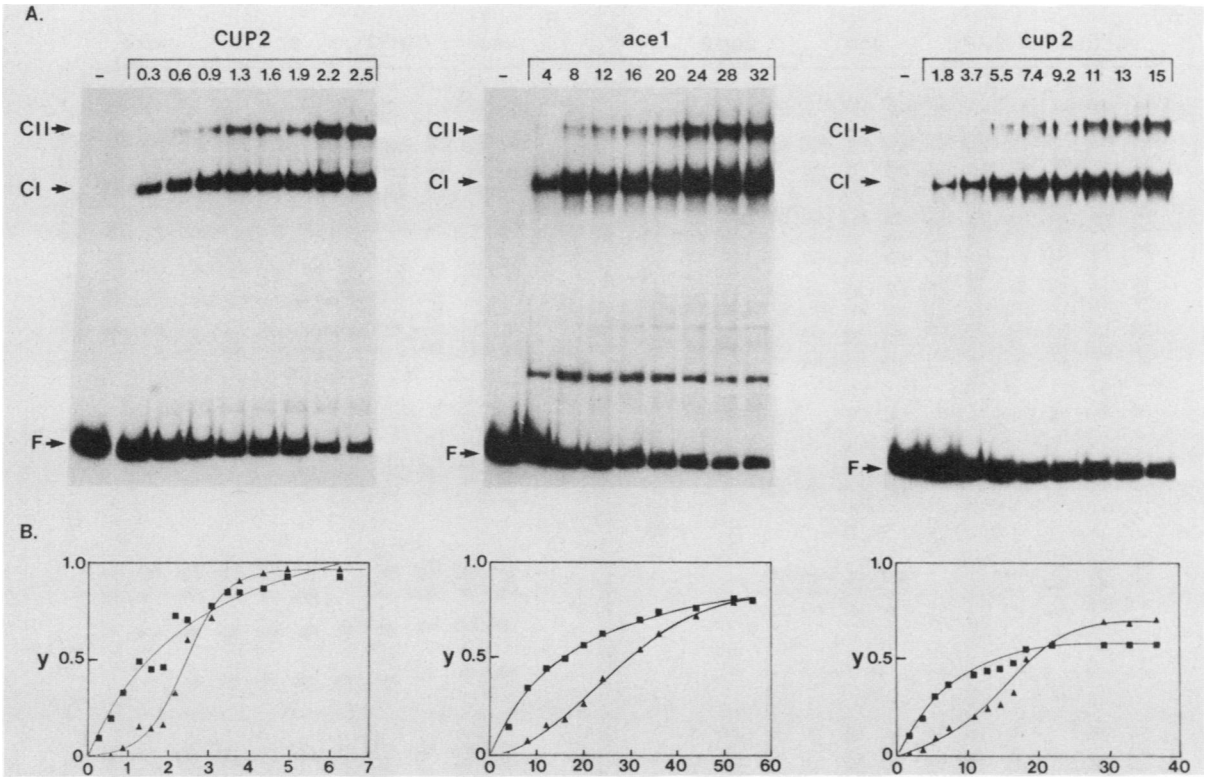

FIG. 2. Protein titrations. (A) Mobility shift assays of binding reactions containing a fixed amount of end-labeled UASc probe (0.3 ng) incubated with increasing amounts of the different CUP2 proteins, as indicated (in nanograms). F, Free UASc probe; CI, complex I; CII, complex II. (B) The mobility shift experiments were quantitated by counting the dried gels with the Ambis radioanalytic imaging system. Amounts of protein added (in nanograms) were plotted against y, the fraction of probe present in complex I (■) or complex II (▲), with each complex analyzed independently. Note that the titrations shown here include amounts of protein for which the raw data are not shown in panel A, owing to space limitations.

additional contacts seen here, probably because they examined a complex equivalent to complex I. The results described above are consistent with the notion that complex I contains one molecule of CUP2 preferentially bound to the UASc between −127 and −142, and complex II consisting of two molecules of CUP2 bound to the entire UASc from −108 to −142. The boundaries of complex II determined by methylation interference are consistent with the borders of the DNase I footprints.

Similar to the observations made by DNase I footprinting, methylation interference indicated that ace1 interacts with a different and smaller part of the UASc. Formation of ace1-UASc complex I was inhibited by methylation of G-117 on the top strand (Fig. 4A) and G-116 and G-119 on the bottom strand (Fig. 4B). It is noteworthy that the effect of methylation on the formation of ace1-UASc complex I was more pronounced than the interference with the formation of CUP2-UASc complex I. Formation of the slower-moving ace1-UASc complex II was strongly inhibited by methylation of G-128 and G-131 on the top strand (Fig. 4A) and G-120 and G-130 on the bottom strand (Fig. 4B), in addition to the G residues that interfered with complex I. Together with the DNase I protection data, these findings are consistent with binding of an ace1 monomer to a site whose minimal borders span G-116 and G-119, whereas two ace1 molecules cover the region between −116 and −131. Hence, in addition to its decreased affinity, the ace1 monomer prefers a different site than a CUP2 monomer. Complete occupancy of the UASc by ace1 leads to protection of only half of the region protected by complete occupancy of the UASc with CUP2. Since cup2 generated an identical DNase I footprint to CUP2, its binding was not further examined by methylation interference.

To further examine the mode of binding by the wild-type protein, we used hydroxyl radical footprinting (39). This method, which identifies sugar residues in the DNA backbone that are protected from hydroxyl radical cleavage, generates additional information regarding protein-DNA contacts not available by the methylation interference method. The hydroxyl radical footprint of CUP2 complex II is shown in Fig. 5. The footprint patterns on the two strands are highly symmetrical around the axis of the pseudodyad (see Fig. 6A) of the UASc centered at position −124. CUP2 protects three regions of each strand, with the most highly protected positions occurring at −133, −127, and −112 (top strand) and −136, −121, and −115 (bottom strand). The two sets of protections at the extremities of the binding site are offset from each other by three bases in the 3′ direction from one strand to the other, indicating that CUP2 crosses the minor groove of the DNA at these points (39). The protection at the center has different symmetry properties. The most highly protected positions here are offset by 6 bases in the 5′ direction, indicative of a major groove interaction, and the protection extends for most of a turn of DNA around the dyad.

We therefore interpret the hydroxyl radical footprint of CUP2 as suggesting that each molecule of the protein crosses the minor groove between the two regions of the major grooves in each half of the UASc where methylation interference detects contacts with guanines. At the center of

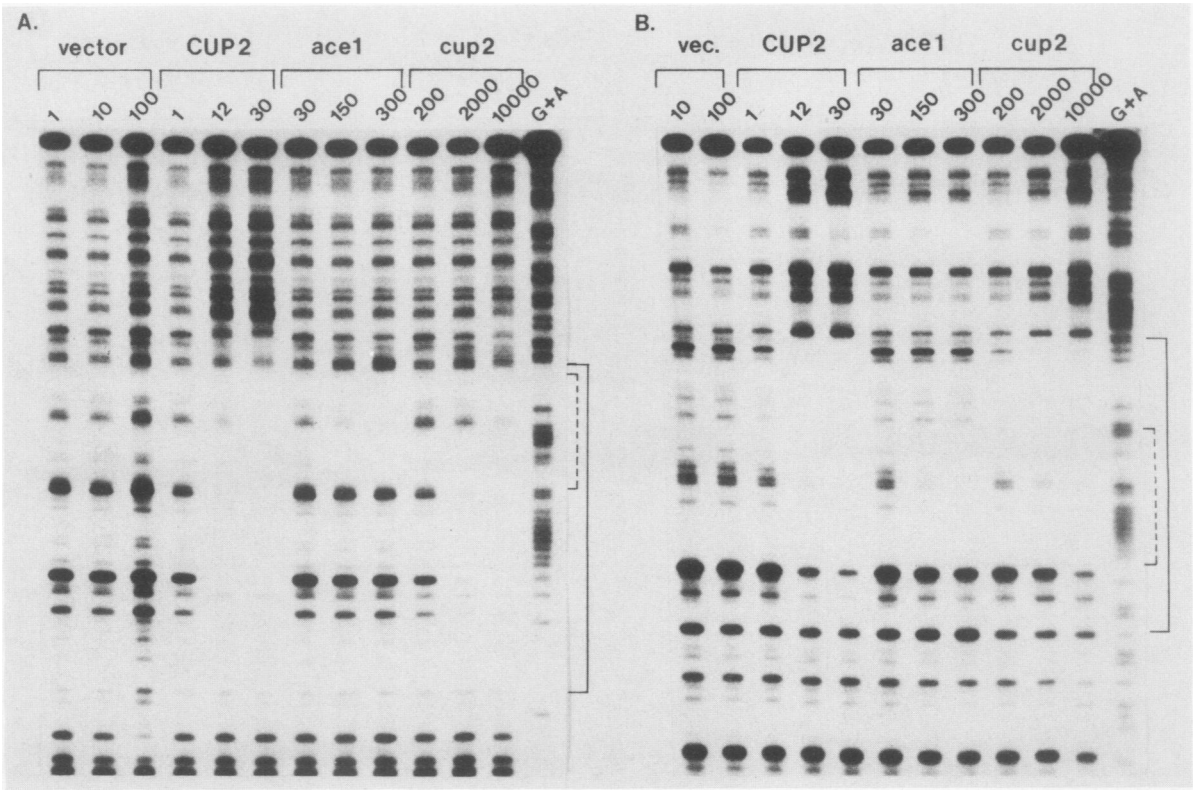

FIG. 3. DNase I footprints of the *CUP1* promoter region. Footprinting reactions were carried out with either the bottom strand (A) or top strand (B) of the *CUP1* promoter and the indicated amounts of purified CUP2 proteins (in nanograms) or with an extract of BL21(DE3) cells transformed with the insertless expression vector (vec). G+A, Maxam-Gilbert sequencing reactions. Solid vertical lines indicate regions of protection from DNase I cleavage by the CUP2 and cup2 proteins. Dashed vertical lines indicate regions protected from DNase I cleavage by the ace1 protein.

the binding site the CUP2 molecule apparently makes contact with the sugar-phosphate backbone on the other side of the DNA molecule, probably by wrapping around the DNA near the dyad center. Since no contacts with guanines were found by methylation interference in the major groove at the dyad center, the interaction with this part of the binding site appears to involve solely backbone contacts. The results of the footprinting and interference experiments are mapped onto a schematic of the DNA helix in Fig. 6B.

Transcriptional activity of CUP2 proteins. To examine the consequences of the reduced DNA-binding activity on the ability of the ace1 and cup2 variants to stimulate transcription of the *CUP1* gene, in comparison with the wild-type CUP2 protein, in vitro transcription experiments were conducted. The purified proteins were added to HeLa whole-cell extract and examined for their ability to stimulate transcription of constructs containing either *CUP1* promoter sequences from −183 to −83 (CUP1-TK) or a synthetic oligonucleotide spanning sequences from −145 to −105 (UASc-TK) upstream to a TK promoter deleted of its Sp1 and CTF sites (Δ5′-46). In the absence of CUP2, both templates were transcribed at a low basal level, equivalent to that of the truncated TK promoter (Fig. 7). Addition of 0.1 μg of CUP2 protein, although suboptimal, led to significant stimulation of transcription from the UASc-containing templates. In the case of the CUP1-TK construct, transcription was initiated from both the authentic TK start site and an upstream site located 30 bp downstream of a TATA sequence present within the *CUP1* DNA. Although addition of increasing amounts of CUP2 led to a further increase in transcription from both start sites, it had a more pronounced effect on the upstream site. These results show that CUP2 expressed in *E. coli* can activate transcription in a UASc-dependent manner in a mammalian extract. The minimal UASc fused to the TK promoter (UASc-TK) was able to confer CUP2-dependent transcription (Fig. 7B) similar to that conferred by the entire *CUP1* upstream region from −83 to −183. At the highest concentration tested, CUP2 led to weak activation of the truncated TK promoter, possibly by binding to low-affinity sites in the vector. The requirement for rather high levels of CUP2 for optimal stimulation is explained by the results of mobility shift assays performed under conditions similar to those of the transcription assay, which indicated a 10-fold drop in binding activity of all three proteins in comparison with the conditions used in the binding assays shown in Fig. 2 (data not shown). Although 0.1 μg of CUP2 led to a significant and reproducible stimulation of transcription, addition of a 10-fold-larger amount of cup2 or ace1 was ineffective.

According to the DNase I footprinting analysis (Fig. 3), ace1 is 10-fold less active than the wild-type CUP2 protein, whereas cup2 is 200-fold less active in DNA binding. Smaller differences in DNA-binding activities were detected by the mobility shift assay (Fig. 2). On the basis of the footprint assay, the transcriptional inactivity of cup2 can be entirely explained by its weak DNA-binding activity. However, the 10-fold reduction in DNA-binding activity, which is also seen under conditions similar to those in the transcription assay (data not shown), does not account for the larger drop in transcriptional activity exhibited by ace1. Hence, the

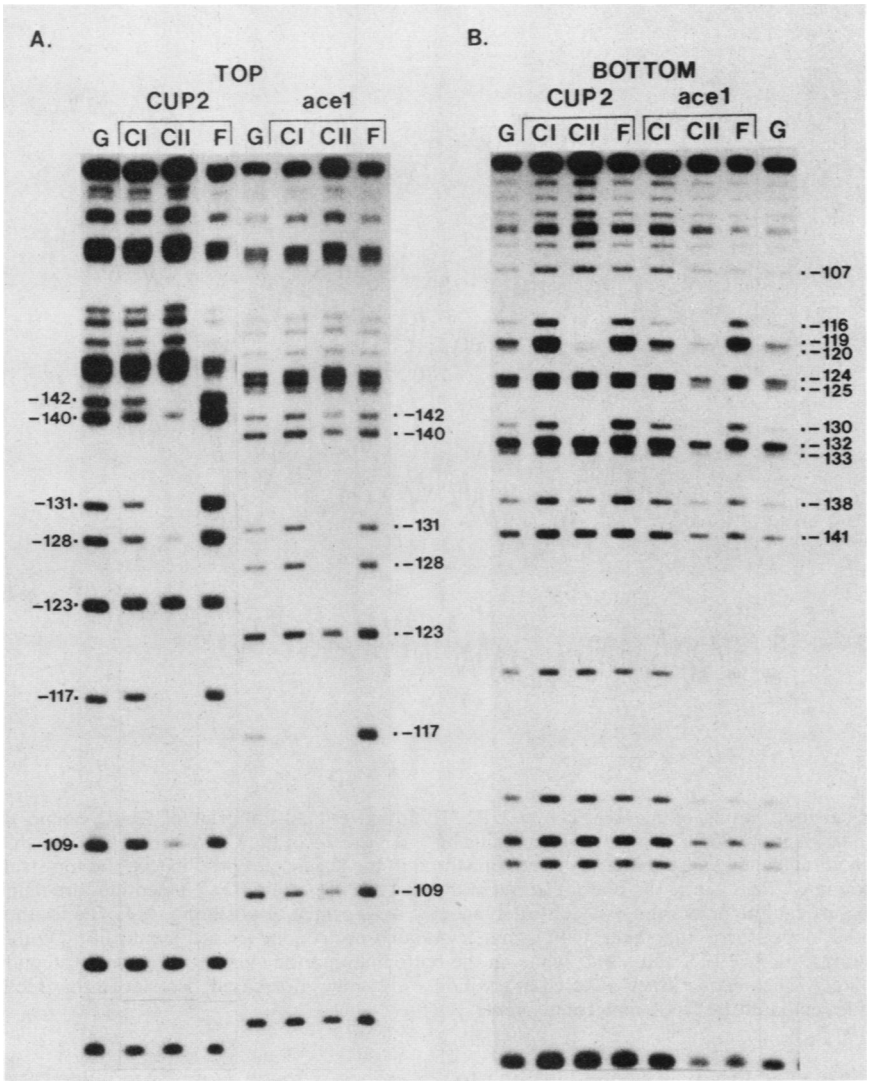

FIG. 4. Methylation interference analysis. The UASc was labeled on either the top (A) or bottom (B) strand and partially methylated with dimethyl sulfate. The probes were used in mobility shift assays with the CUP2 and ace1 proteins. Complex I (CI), complex II (CII), and free (F) DNAs were eluted from the gels, cleaved with piperidine, and electrophoresed through a 10% sequencing gel. The G-residue positions within the UASc are indicated.

altered manner of DNA binding may also cause a defect in transcriptional activation by ace1. These results match the in vivo findings, indicating that the *CUP1* gene is completely refractory to Cu induction in both *cup2* and *ace1-1* strains (36, 42).

Interestingly, transcription of the α-globin template used as an internal control was not affected by addition of up to 0.5 μg of CUP2, 1 μg of cup2, or 1 μg of ace1, respectively. Addition of 1 μg of CUP2 led to a large decrease in α-globin transcription, while enhancing CUP1-TK transcription (Fig. 7A). This effect may be similar to the squelching effect, previously described by Gill and Ptashne (15). However, for CUP2 this presumed competition for general transcription factors appears to require binding to DNA, as the ace1 and cup2 proteins did not show such an effect at the levels tested in the assay.

DISCUSSION

The results of the different DNA-binding assays, summarized in Fig. 6, are consistent with the following description of the interaction of CUP2 with the UASc. Examination of the UASc sequence reveals an extensive palindrome whose half-site is 5′-GTCTTTTPyPyGCTGAAC-3′. We also observed that the entire binding site (−140 to −107) can be considered to be palindromic around a dyad center at position −124, if the G · C base pair at position −120 is eliminated (Fig. 6A). The hydroxyl radical footprint is symmetrical about this pseudodyad, supporting this view of the UASc. A monomer of CUP2 first binds to the 5′ half-site present between −140 and −128, followed by a second molecule binding to the 3′ half-site between −107 and −115. Each half-site is spread over one and one-half turns of the double helix and contains residues whose methylation interferes with CUP2 binding. These residues are separated by one complete turn and face the same side of the helix. The sugars that line the minor groove in the center of the half-site are protected by CUP2 from hydroxyl radical cleavage. Together, these observations suggest that the protein is lying on top of the minor groove, contacting the major groove in two different sites. In addition, each of the monomers seems

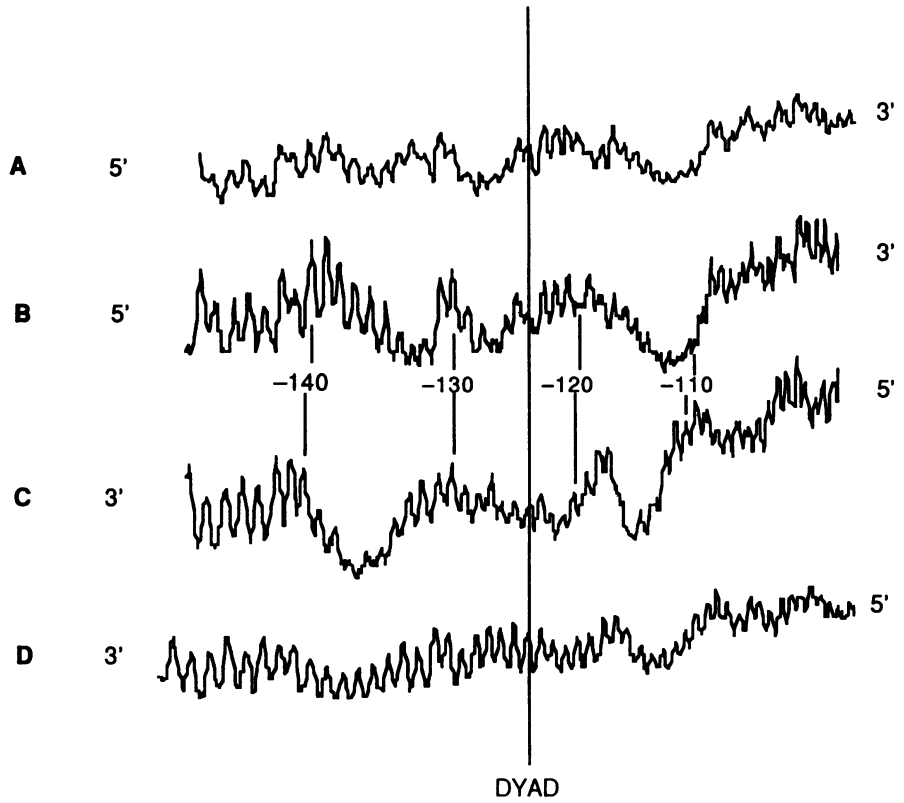

FIG. 5. Hydroxyl radical footprinting of the UASc by CUP2. Hydroxyl radical footprint of CUP2 bound to the UASc. The 154-bp *Hin*dIII-*Eco*RI restriction fragment used for this footprint contains bp −183 to −83 of the CUP1 promoter. Shown are densitometer scans of autoradiographs of hydroxyl radical cleavage patterns on the top strand in the absence of protein (A), the top strand with bound CUP2 (B), the bottom strand with bound CUP2 (C), and the bottom strand in the absence of protein (D). Nucleotides are numbered relative to the start site of transcription. The vertical line marks the pseudodyad symmetry axis located at position −124. The footprint is clearly symmetrical from one strand to the other about the pseudodyad, with symmetry-related protections shaped identically. On the top strand the strongest protections occur at positions −133, −127, and −112, while on the bottom strand the symmetry-related protections are at positions −136, −121, and −115, respectively. The adenine tract centered at position −112 shows decreased cleavage in the free DNA samples (A and D), perhaps indicating that this region of the DNA molecule is bent.

to wrap around the DNA near the dyad center, leading to protection of sugars on the back side of the helix. The interaction of the ace1 variant with the UASc is, however, entirely different. An ace1 monomer first binds to a portion of the downstream half-site located between positions −116 and −120, followed by binding of a second molecule to a portion of the upstream half-site, between −127 and −131. From Fig. 6B it is evident that each of the ace1 half-sites corresponds to only the innermost part of the CUP2 half-site.

These findings are consistent with the conclusion that CUP2 has either a bipartite DNA-binding domain or an extended DNA-binding domain consisting of several elements, each interacting with a different part of the recognition sequence. One of the recognition elements of CUP2 interacts with the outermost part of each half-site, while the other DNA-binding elements interact with the innermost part of each half-site. The Cys-11-to-Tyr substitution of ace1 presumably inactivates the DNA-binding element responsible for contacting the outermost part, and therefore this variant protein can interact only with the innermost part of the half-site. In addition, ace1 exhibits a different sequence preference from the CUP2 protein. The ace1 monomer first binds to the 3′ binding site, whose sequence is 5′-TGCTG GAAC-3′, whereas the CUP2 monomer prefers the 5′ binding site, 5′-GTCTTTTCCGCTGAAC-3′. The sequence preference is much more pronounced for ace1, as indicated by the stronger effect of methylation of distinct G residues on formation of the monomeric complex. On the other hand, the less distinct effect of the G methylations on formation of the monomeric CUP2 complex suggests that some of this complex also contains protein bound to the 3′ site.

CUP2 is a Cu-dependent DNA-binding protein (4, 11). The presence of 12 cysteine residues within its DNA-binding domain suggested that the active DNA-binding domain consists of a cysteine-coordinated Cu cluster (11). In support of this hypothesis, we found that substitution of Cys-11 by a Tyr residue generates a protein that contains one less Cu atom than the wild-type protein (five versus six Cu atoms per molecule, respectively). This substitution also leads to inactivation of part of the DNA-binding domain. This variant DNA-binding domain is still functional, as indicated by the ability of ace1 to bind DNA in a sequence-specific manner. The analysis described here leads to a refinement of the Cu cluster hypothesis (10) and suggests that folding of the CUP2 DNA-binding domain around the Cu^{+} ions leads to formation of an extensive protein domain in which different regions interact with separate parts of the recognition site. Further understanding of the mode of interaction between CUP2 and the DNA will require determination of the tertiary structure of its DNA-binding domain. The availability of

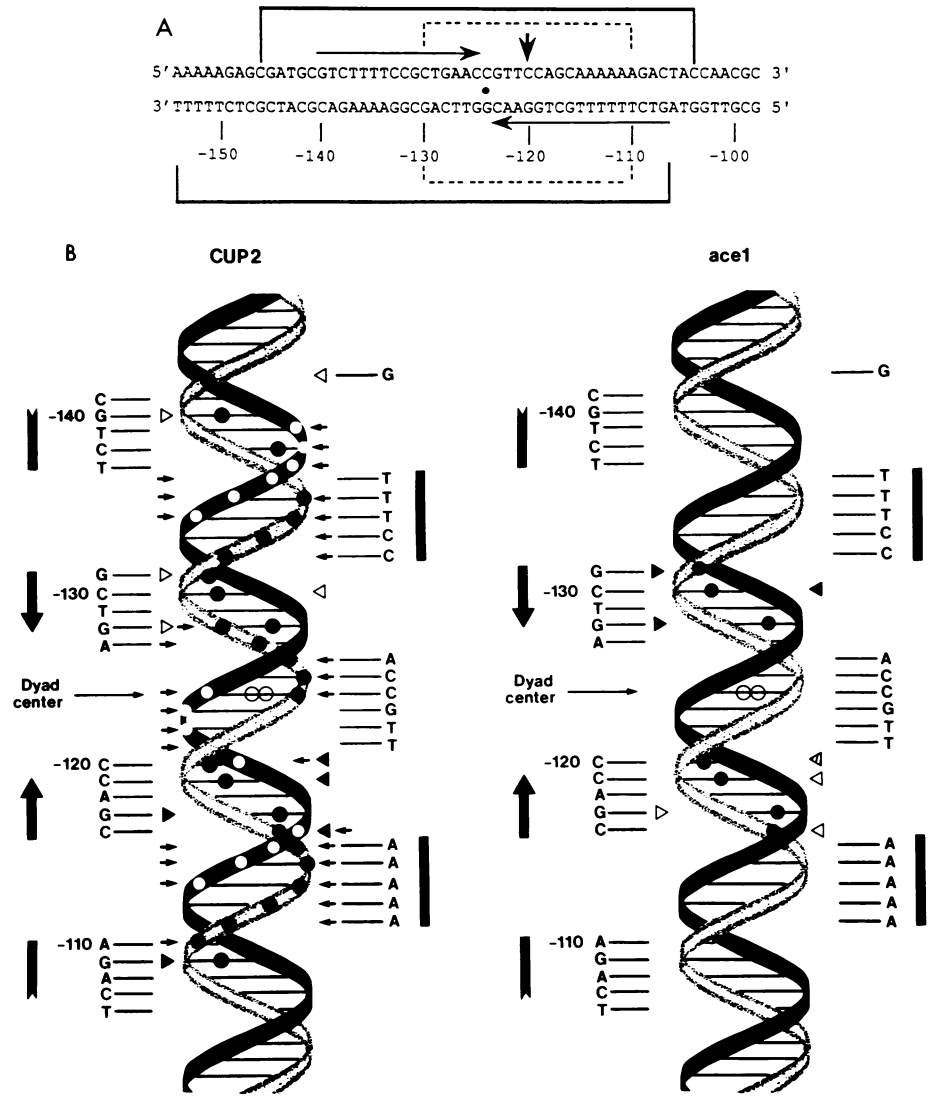

FIG. 6. Interaction of CUP2 and ace1 with the UASc. (A) Sequence of the UASc. Base pairs are numbered relative to the start site of transcription of the CUP1 gene. Horizontal arrows indicate the region of almost perfect dyad symmetry. The dot in the center is placed at position −124, the center of the pseudodyad. The vertical arrow marks the G · C base pair at position −120 that interrupts the perfect dyad symmetry (see text). Brackets above and below the sequence demarcate the DNase I footprints of CUP2 (solid line) and ace1 (broken line) on the UASc. (B) The results obtained by the various protection assays are summarized on a 10.5-bp-per-turn double-helical presentation of the UASc. Symbols: ▷, G residues whose methylation interferes with formation of complex I; ▶, additional G residues whose methylation interferes with formation of complex II; →, hydroxyl radical protections. In addition, the base pairs whose G methylation interferes with binding, and the sugars protected against hydroxyl radical cleavage, are indicated by circles. The two halves of the UASc palindrome are marked by the vertical arrows, and the dyad center is indicated by the double circle.

large amounts of functional recombinant CUP2 protein should facilitate such studies.

Different parts of the CUP2 DNA-binding domain may make their own distinct contacts with the DNA and recognize different sequence motifs, as suggested by the comparison of the CUP2 and ace1 proteins. It is therefore possible that other metal-coordinated DNA-binding proteins that interact with relatively large binding sites will also use different parts of their DNA-binding domain, each one having a distinct specificity, to recognize their complex target sites. This suggestion is indeed consistent with the deletion analysis of TFIIIA, which indicated that different clusters of Zn fingers are used to contact different parts of the recognition site (41). The ability of each element to interact with the DNA independently of the other elements may be important for allowing such proteins to remain anchored to the DNA even during the progression of the replicational or transcriptional machinery through their binding sites. Although it has not been shown yet that CUP2 can stimulate transcription after binding downstream of the initiation site, this is a known property of TFIIIA (30, 43) and steroid hormone receptors (33). This modular composition of DNA-binding domains may be an important contributor to the evolution of such proteins. As shown above, a single-amino-acid change does not lead to complete loss of DNA-binding activity but instead generates a protein with modified site specificity. If such mutations occur after amplification of the original gene, they can generate a family of structurally similar DNA-binding proteins with distinct target site specificities.

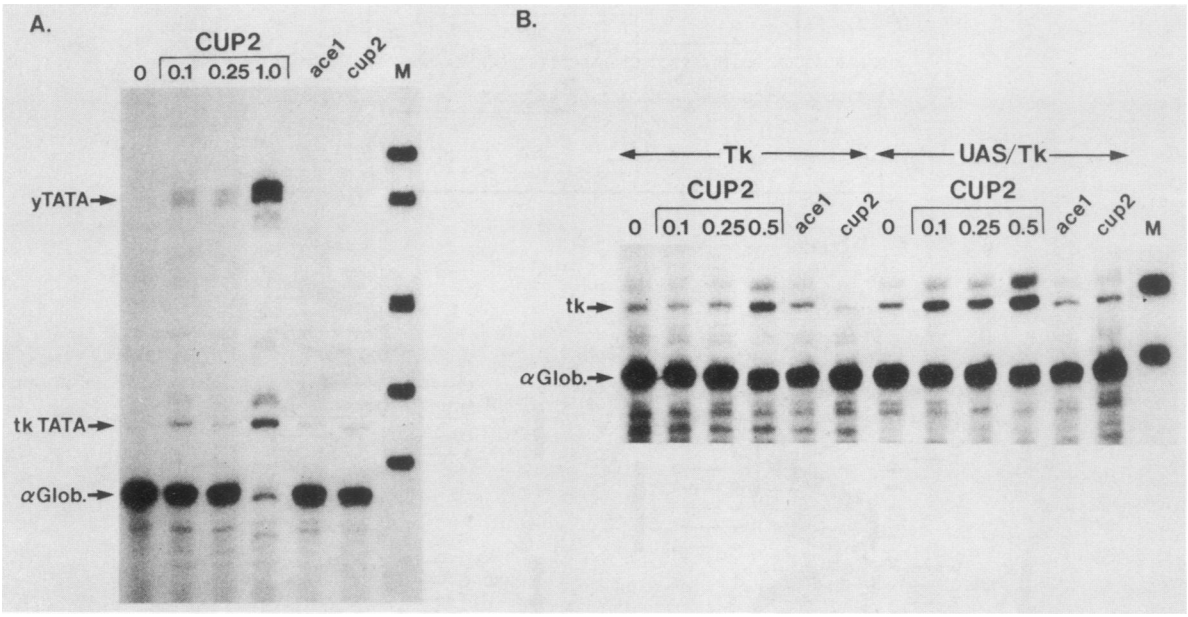

FIG. 7. Transcriptional activation of CUP1-TK hybrid promoters by CUP2. (A) Effect of added CUP2 (in micrograms) ace1 (1 μg), and cup2 (1 μg) on the transcriptional efficiency of the CUP1 TK template (200 ng) in HeLa whole-cell extracts. The human α-globin gene (50 ng) was included as an internal control. cDNAs corresponding to transcripts initiated at the correct TK start site (tkTATA), at a site downstream to a TATA sequence in *CUP1* DNA (yTATA), and the α-globin promoter (αGlob.) are indicated. *Hpa*II fragments of pBR322 were used as markers. (B) Effect of CUP2 (in micrograms), ace1 (1 μg), and cup2 (1 μg) on the transcriptional efficiency of UASc TK (200 ng), Δ5′-46TK (200 ng), and human α-globin (50 ng) templates. cDNAs corresponding to transcripts initiated at the TK and α-globin promoters are indicated.

Although the ace1 protein can still bind to the UASc, this interaction does not result in transcriptional activation either in vivo (36) or in vitro. The in vitro defect was observed even after addition of amounts of ace1, giving the same fractional occupancy of the UASc as a smaller amount of the wild-type CUP2 protein which is sufficient for stimulating transcription in a UASc-dependent manner. It remains to be tested, however, whether ace1 binds to the UASc in vivo as shown for CUP2 (18). Nevertheless, these findings raise the possibility that proper interaction of CUP2 with the DNA is required not only for stabilizing the protein-DNA complex but also for generating either a complex of a particular spatial arrangement consistent with transcriptional activation or a stable complex between CUP2 and the initiation machinery. Similar observations were made for the HAP1 activator (20) and the glucocorticoid receptor (16). Such observations suggest that in addition to specialized activation domains (28), DNA-binding domains may play an active role in interactions with the transcriptional machinery. Indeed, it was recently demonstrated that in vitro the CUP2 DNA-binding domain alone may be sufficient for a low-level transcriptional activation (5).

ACKNOWLEDGMENTS

We thank P. Geiduschek for critical comments, F. W. Studier for expression vectors and strains, M. Hattori for instruction on methylation interference, P. Saltman and B. Dyke for atomic absorption spectroscopy, L. Defize and T. Smeal for helpful discussion, J. Meek for artwork, and A. Casey for typing.

P. Skroch was supported by a postdoctoral fellowship from the Deutscher Akademischer Austauschdienst. This research was supported by grants from the National Institutes of Health and the U.S. Environmental Protection Agency.

LITERATURE CITED

1. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell **49**:729–739.
2. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56**:335–344.
3. **Berg, J. M.** 1989. DNA binding specificity of steroid hormone receptors. Cell **57**:1065–1068.
4. **Buchman, C., P. Skroch, J. Welch, S. Fogel, and M. Karin.** 1989. The *CUP2* gene product, regulator of yeast metallothionein expression, is a copper-activated DNA-binding protein. Mol. Cell. Biol. **9**:4091–4095.
5. **Culotta, V. C., T. Hsu, S. Hu, P. Furst, and D. Hamer.** 1989. Copper and the ACE1 regulatory protein reversibly induce yeast metallothionein gene transcription in a mouse extract. Proc. Natl. Acad. Sci. USA **86**:8377–8381.
6. **Danielson, M., L. Hinck, and G. M. Ringold.** 1989. Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell **57**:1131–1138.
7. **Dinter, N., R. Chiu, M. Imagawa, M. Karin, and K. A. Jones.** 1987. In vitro activation of the HIV-1 enhancer in extracts from cells treated with a phorbol ester tumor promoter. EMBO J. **6**:4067–4071.
8. **Evans, R. M., and S. M. Hollenberg.** 1988. Zinc fingers: gilt by association. Cell **52**:1–3.
9. **Fried, M., and A. D. Crothers.** 1981. Equilibrium and kinetics of lac repressor-operator interaction by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
10. **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.
11. **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.
12. **Galas, D. J., and A. Schmitz.** 1978. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
13. **Garner, M., and A. Rezvin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions:

application to components of the *E. coli* lactose regulatory system. Nucleic Acids Res. **9**:3047–3060.
14. **Gibson, T. J., J. P. M. Potsma, R. S. Brown, and P. Argos.** 1988. A model for the tertiary structure of the 28-residue DNA-binding motif (zinc finger) common to many eukaryotic transcription regulatory proteins. Protein Eng. **2**:209–218.
15. **Gill, G., and M. Ptashne.** 1988. Negative effect of the transcriptional activator GAL4. Nature (London) **334**:721–724.
16. **Hollenberg. S. M., and R. M. Evans.** 1988. Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. Cell **55**:899–906.
17. **Hope, I. A., and K. Struhl.** 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell **46**:885–894.
18. **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 **86**:65–69.
19. **Karin, M., R. Najarian, A. Haslinger, J. Welch, P. Valenzuela, and S. Fogel.** 1984. Primary structure and transcription of an amplified genetic locus: the *CUP1* locus of yeast. Proc. Natl. Acad. Sci. USA **81**:337–341.
20. **Kim, K. S., and L. Guarente.** 1989. Mutations that alter transcriptional activation but not DNA binding in the zinc finger of yeast activator HAP1. Nature (London) **342**:200–203.
21. **Klug, A., and D. Rhodes.** 1987. Zinc fingers: a novel protein motif for nucleic acid recognition. Trends Biochem. Sci. **12**:464–469.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Lee, M. S., G. P. Gippert, K. V. Soman, D. A. Case, and P. E. Wright.** 1989. Three-dimensional solution structure of a single zinc finger DNA-binding domain. Science **245**:635–637.
24. **Marder, S., V. Kumar, H. de Verneuil, and P. Chambon.** 1989. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid responsive element. Nature (London) **338**:271–274.
25. **McCormick, A., D. Wu, J. L. Castrillo, S. Dana, J. Strobl, E. B. Thompson, and M. Karin.** 1988. Extinction of growth hormone expression in somatic cell hybrids involves repression of the specific trans-activator GHF-1. Cell **55**:379–389.
26. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domain in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. **4**:1609–1614.
27. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53**:293–321.
28. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335**:683–689.
29. **Rhodes, D., and A. Klug.** 1986. An underlying repeat in some transcriptional control sequences corresponding to half a double helical turn of DNA. Cell **46**:123–132.
30. **Sakonju, S., D. F. Bogenhagen, and B. B. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription. I. The 5′ border of the region. Cell **19**:13–25.
31. **Severnne, Y., S. Wieland, W. Schaffner, and S. Rusconi.** 1988. Metal binding "finger" structures in the glucocorticoid receptor defined by site-directed mutagenesis. EMBO J. **7**:2503–2508.
32. **Siebenlist, U., and W. Gilbert.** 1980. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T7. Proc. Natl. Acad. Sci. USA **77**:122–126.
33. **Slater, E. P., O. Rabenau, M. Karin, J. D. Baxter, and M. Beato.** 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by the first intron of the human growth hormone gene. Mol. Cell. Biol. **5**:2984–2992.
34. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54**:855–864.
35. **Szczypka, M. S., and D. J. Thiele.** 1989. A cysteine-rich nuclear protein activates yeast metallothionein gene transcription. Mol. Cell. Biol. **9**:421–429.
36. **Thiele, D. J.** 1988. *ACE1* regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. Mol. Cell. Biol. **8**:2745–2752.
37. **Tsai, S. Y., M. J. Tsai, and B. W. O'Malley.** 1989. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. Cell **57**:443–448.
38. **Tullius, T. D., and B. A. Dombroski.** 1985. Iron (II) EDTA used to measure the helical twist along any DNA molecule. Science **230**:679–681.
39. **Tullius, T. D., and B. A. Dombroski.** 1986. Hydroxyl radical "footprinting": high-resolution information about DNA-protein contacts and application to repressor and cro protein. Proc. Natl. Acad. Sci. USA **83**:5469–5473.
40. **Unesono, K., and R. M. Evans.** 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell **57**:1139–1146.
41. **Vrana, K. E., M. E. A. Churchill, T. D. Tullius, and D. D. Brown.** 1988. Mapping functional regions of transcription factor IIIA. Mol. Cell. Biol. **8**:1684–1696.
42. **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.
43. **Wolffe, A., P. E. Jordan, and D. D. Brown.** 1986. A bacteriophage RNA polymerase transcribes through a Xenopus 5S RNA gene transcription complex without displacing it. Cell **44**:381–389.