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Copper and the ACE1 regulatory protein reversibly induce yeast metallothionein gene transcription in a mouse extract

(metal ions/transcription factor/DNA-binding protein)

VALERIA CIZEWSKI CULOTTA, TSAO HSU, STELLA HU, PETER FÜRST, AND DEAN HAMER

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **We describe a cell-free system in which the transcription of the yeast metallothionein gene is inducible by the addition of metal ions plus a specific regulatory protein. Efficient transcription requires the complete yeast ACE1 metalloregulatory protein, including both its DNA-binding and transactivation domains; a mouse nuclear extract providing RNA polymerase and general transcription factors; a template containing the ACE1 binding site; and Cu(I). Because the binding of ACE1 to DNA is dependent on Cu, it is possible to inhibit transcription by the use of Cu-complexing agents such as CN^-. We have used this specific inhibition to show that the ACE1 regulatory protein is required for the maintenance as well as the formation of a functional preinitiation complex. The ability to reversibly induce yeast metallothionein gene transcription *in vitro* provides a powerful system for determining the molecular mechanism of a simple eukaryotic regulatory circuit.**

Gene transcription can be activated or repressed by a variety of small effector molecules such as nutrients, hormones, second messengers, and metal ions. In prokaryotes, such effector molecules often act directly by binding to transcriptional activator or repressor proteins. In eukaryotes, the mechanisms of signal transduction are less clear but often appear to involve complex metabolic pathways as well as direct interactions with transcription factors. To understand how effector molecules ultimately affect transcription, it is crucial to develop biochemically defined *in vitro* systems in which they are active. Several crude or partially purified eukaryotic systems have been described in which transcription can be activated by either general or gene-specific regulatory proteins (1, 2); however, none of these appears to be sensitive to the addition of small effector molecules. Here we describe the development of such a system by use of the metal-regulated yeast metallothionein gene, *CUP1*.

The yeast *CUP1* gene encodes a small, metal-binding protein that protects cells against Cu toxicity (3–5). The transcription of this lower eukaryotic metallothionein gene is strongly induced by the presence of excess Cu (or Ag) ions in the growth medium (6, 7). This form of regulation is mediated by a trans-acting factor encoded by the *ACE1* locus (8), also known as the *CUP2* locus (9). Although the *ACE1* gene is constitutively expressed in either the absence or the presence of metal ions (10, 11), the apoprotein cannot bind to DNA (11). In the presence of Cu(I), or the analogous Ag(I) ion, the amino-terminal domain of ACE1 undergoes a conformational switch into a folded, protease-resistant form that can specifically recognize the *CUP1* upstream activator sequence (UAS) both *in vitro* (11) and *in vivo* (12). The amino-terminal domain of ACE1 (amino acids 1–122) is unusually rich in cysteines and basic amino acid residues. Based on similarities of ACE1 to metallothionein itself, we proposed that the amino-terminal domain of ACE1 forms a cysteine-Cu(I) copper-cluster structure upon which are superimposed specific DNA-binding loops (11). The carboxyl-terminal domain of ACE1, which lacks cysteine residues, is extremely acidic and thus resembles the transactivation domains described in several eukaryotic gene activation proteins (13–15). While it has been shown that this carboxyl-terminal region is important for *CUP1* gene regulation *in vivo* (11), its precise role in transcriptional activation was not determined.

To understand how ACE1 activates transcription once it has bound to the *CUP1* gene, we wished to develop an *in vitro* transcription system responsive to ACE1 and Cu ions. Yeast and mammalian transcription factors can interact with one another both *in vivo* (16–19) and *in vitro* (20), and ACE1 can stimulate the transcription of a cotransfected *CUP1* gene in mouse cells (T.H. and D.H., unpublished results). These results prompted us to attempt regulation of the *CUP1* gene in a cell-free system derived from mouse. An important motivation for these experiments was the realization that since ACE1 requires Cu to bind to DNA, it might be possible to specifically inhibit transcription by Cu-complexing agents. Using such an inhibitor, we show that ACE1 is required for *CUP1* gene transcription even after the formation of a sarkosyl-resistant, committed preinitiation complex.

MATERIALS AND METHODS

Nuclear extracts were prepared from exponentially growing mouse L cells by a slight modification of the method of Parker and Topol (21). The extraction buffers contained 0.2 mM phenylmethanesulfonyl fluoride and 1 mM sodium metabisulfite as protease inhibitors, and the dialysis buffer contained 57 mM $(NH_4)_2SO_4$ and 1 mM $MgCl_2$ in place of KCl.

In vitro transcription mixtures (20 μl) contained 4 μl of mouse nuclear extract, 100 ng of supercoiled template DNA, 0.5–2.0 μg of supercoiled pUC DNA carrier (amount required varied with extract preparation), and 500 μM each rATP, rUTP, rGTP, and rCTP, 16 units of RNasin (Promega) in 12.5 mM Hepes, pH 7.9/7.5 mM $MgCl_2$/20 mM $(NH_4)_2SO_4$/10% (vol/vol) glycerol/2 mM dithiothreitol. Reaction mixtures were also supplemented with Cu^I-acetonitrile, other metals, KCN, or sarkosyl (*N*-lauroylsarcosine), as specified, and the indicated amounts of a wheat germ translation mixture (New England Nuclear) programmed with *ACE1* mRNA or mutant derivatives (11). The amount of ACE1 peptide in the wheat germ extract was determined by trichloroacetic acid precipitation and confirmed by SDS/PAGE. After a 60-min incubation at 30°C, transcription reactions were terminated by adding 300 μl of 200 mM NaCl/20 mM EDTA/1.0% SDS containing yeast tRNA at 100 μg/ml, and nucleic acids were purified by successive extrac-

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Abbreviations: UAS, upstream activator sequence; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase.

tions with phenol and chloroform and by precipitation at room temperature with 2-propanol. The amount and initiation site of the transcripts were then determined by primer extension (22).

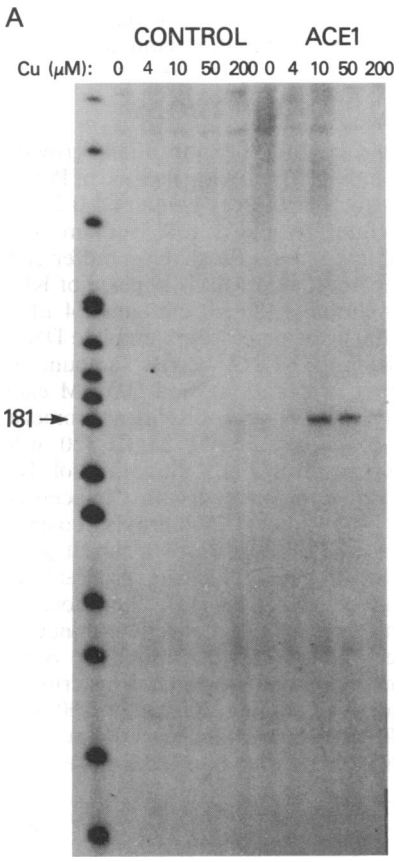
All templates were constructed by inserting yeast or mouse metallothionein gene sequences into the polylinker sites of p8CAT (22), a vector containing the bacterial chloramphenicol acetyltransferase (CAT) gene. Plasmid CUP1:CAT contains *CUP1* nucleotides −400 to +30 (for numbering system see ref. 23). Plasmid UASc:yTATA:CAT carries an oligonucleotide corresponding to the *CUP1* UASc control region (nucleotides −105 to −148) fused to the *CUP1* TATA and initiation sequences (nucleotides −112 to +30; see ref. 11). The yTATA:CAT construct contains only the *CUP1* TATA and initiation region. Plasmid UASc:mTATA:CAT carries the UASc oligonucleotide fused to the mouse metallothionein I gene TATA element and initiation region (residues −34 to +18) of 5′Δ34CAT (22). The construction of mTATA:CAT (equivalent to 5′Δ34CAT) and mMT-I:CAT (equivalent to p8MTCAT) has been described (22).

RESULTS

ACE1- and Copper-Dependent Transcription Initiation of the *CUP1* Gene in Mouse Nuclear Extract. To study the regulation of the yeast metallothionein gene *in vitro*, we derived a cell-free system consisting of the following components: (*i*) a supercoiled template containing *CUP1* gene upstream regulatory elements linked to a CAT reporter gene: (*ii*) a mouse nuclear extract, prepared by standard methods, to provide general transcription factors and RNA polymerase II (21); (*iii*) ACE1 protein, or derivatives thereof, prepared by translation in a wheat germ extract (11); and (*iv*) metal ions. Following transcription, the amount and initiation site of the RNA were determined by primer extension using a CAT oligonucleotide (22).

Fig. 1*A* shows an experiment in which template CUP1:CAT was incubated in the mouse extract in the presence of control or ACE1 wheat germ extract and various concentrations of Cu(I). This template contains the complete *CUP1* gene promoter (−400 to +30), including the yeast TATA box and initiation region and the UASc/UASd upstream regulatory region, which contains two ACE1 binding sites (11, 23). In the absence of ACE1, or in the presence of ACE1 but absence of Cu(I), a low background level of transcription was observed; it is unknown whether this reflects a low level of authentic initiation in the absence of active ACE1 or an artifact of the primer extension assay, since several other minor bands are observed elsewhere in the autoradiogram of the gel. The addition of both ACE1 and 10–50 μM Cu(I) resulted in a strong increase in the transcription of a CUP1–CAT fusion RNA. The ratio of specific to nonspecific transcription was >25 in the experiment shown in Fig. 1*A* and ranged from 10 to 30 in other experiments. The addition of 200 μM Cu(I) repressed transcription, reflecting the fact that excess metal inactivates the DNA-binding activity of ACE1 (11), presumably due to destabilization or oxidation of the metal-thiolate cluster (24). These experiments show that both ACE1 and Cu(I) are required for efficient transcription *in vitro*.

Interestingly, the 5′ end of the RNA synthesized *in vitro* maps ≈50 base pairs (bp) upstream of the initiation site used in yeast (Fig. 1*B*). The sequence TATAAA, a perfect match to the mouse metallothionein I gene TATA box, lies 30 bp upstream from the initiation site used in the mouse extract. This is a typical distance between the TATA box and initiation site for mammalian genes. In contrast, two potential TATA sequences for the yeast initiator, TATAAT and TATAAA, lie 33 and 77 bp upstream of the yeast initiation site, with the latter distance being more typical of yeast promoters. Thus, even though the mouse extract responds to the yeast ACE1 regulatory protein, the TATA and initiation



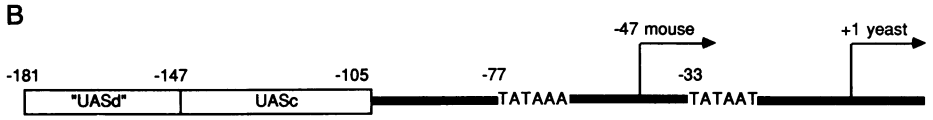


FIG. 1. ACE1- and Cu-dependent transcription of the yeast *CUP1* gene in a mouse nuclear extract. (*A*) *In vitro* transcription of the CUP1:CAT template DNA was tested in reaction mixtures containing the indicated concentrations of Cu^{I}-acetonitrile and 4 μl of either control wheat germ translation mixture or an identical mixture containing *in vitro* translated ACE1 protein (≈3 fmol/μl). Transcripts were quantitated and mapped by primer extension using an oligonucleotide complementary to the CAT gene (22). Arrow marks the 181-nucleotide fusion RNA containing 86 bases from the CAT gene and 95 bases from the yeast *CUP1* gene. (*B*) Map of the *CUP1* promoter showing the transcription initiation site utilized in the mouse extract as compared to the site used in yeast cells. Numbers indicate nucleotide position on the *CUP1* gene relative to the yeast initiation site (23).

factors follow the preferences typical of mammalian organisms.

Transcriptional Induction Requires UAS Control Sequences. To determine whether *in vitro* transcription depends on the binding of ACE1 to the template, we compared the efficiency and inducibility of a series of plasmids that contain or lack an ACE1 binding site (Table 1). Plasmid UASc:yTATA:CAT carries a 45-bp oligonucleotide, which corresponds to a single copy of the UASc control region, fused to the yeast TATA and initiation sites. This construct, which was transcribed from the same initiation site as CUP1:CAT, was clearly inducible by ACE1 and Cu(I) and was transcribed about 40% as well as the complete promoter. This parallels the situation in yeast cells, in which the same synthetic construct is transcribed at 70% the level of the wild-type promoter (11). In contrast, the control construct yTATA:CAT, which contains the yeast TATA box and initiation site but no ACE1 binding site, yielded only background levels of transcription and was not induced by the combination of ACE1 and Cu(I).

To test the effect of TATA and initiation sites on transcription, we constructed plasmid UASc:mTATA:CAT, which carries the same yeast UASc oligonucleotide control sequence fused to the TATA and initiation sequences of the mouse metallothionein I gene. The yeast UAS was placed 43 bp upstream of the mouse TATA box, a spacing similar to that between the first metal response element and the TATA box of the mouse metallothionein I gene (25, 26). This template faithfully initiated transcription at the mouse metallothionein gene start site (see Fig. 3) and gave an induced transcription level ≈2.4-fold higher than that of the complete yeast promoter and an induction ratio of 16 (Table 1). In contrast to the yeast constructs, this mouse-based plasmid also gave detectable background transcription in the absence of ACE1 or Cu(I). In a control experiment, template mTATA:CAT (22), which carries the mouse TATA box and initiation region but no ACE1 binding site, also gave a discernible level of transcription but was not induced by the addition of ACE1 protein and Cu(I). These experiments provide further evidence that the mouse general transcription factors prefer mammalian downstream regulatory sequences.

As a further control for any nonspecific effects of ACE1 or Cu(I) on transcription, we tested the complete mouse metallothionein I gene (mMT-1:CAT), which is inducible by metals in mammalian cells (22) but does not contain an ACE1 binding site and is not metal-inducible in yeast cells (T.H. and D.H., unpublished observations). Although the mouse gene was efficiently transcribed *in vitro* (Table 1), it was not induced by the addition of ACE1 protein or Cu(I); in fact, it appeared to be somewhat repressed. These results confirm that regulation in the mouse extract is mediated by the binding of the ACE1 regulatory protein.

Table 1. Transcriptional efficiencies of various templates in the *in vitro* transcription system

Template	Relative transcription		
	− ACE1, + Cu	+ ACE1, − Cu	+ ACE1, + Cu
CUP1:CAT	<5	<5	(100)
UASc:yTATA:CAT	<5	<5	38 ± 13
yTATA:CAT	<5	<5	<5
UASc:mTATA:CAT	14	15	235 ± 80
mTATA:CAT	18	16	19
mMT-1:CAT	425	225	155

Transcription mixtures (as described in Fig. 1) containing the indicated metallothionein gene template were supplemented with 4 μl of either a control wheat germ translation mixture (− ACE1) or a mixture containing 12 fmol of ACE1 (+ ACE1) and were incubated in the presence (+ Cu) or absence (− Cu) of 25 μM Cu^{I}-acetonitrile. The transcription products were quantitated by densitometry of the relevant autoradiograms and normalized to a value of 100 for the induced activity of CUP1:CAT. The induced activities for UASc:yTATA:CAT and UASc:mTATA:CAT are presented as mean ± SD for four determinations.

Both DNA-Binding and Transactivation Domains of ACE1 Are Important for Regulation *in Vitro*. We next compared the ability of various ACE1 derivatives to direct transcription in the mouse extract. We proposed previously (11) that ACE1 consists of two domains: an amino-terminal DNA- and metal-binding domain that is rich in basic amino acids and cysteines, and a carboxyl-terminal domain that is not required for DNA binding but is similar to many transactivation domains in its high content of acidic residues (11).

A mutant ACE1 protein in which both Cys-88 and Cys-90 are converted to serine residues does not interact with Cu, as shown by a proteolysis protection assay, and therefore cannot bind to DNA (S.H., P.F., and D.H., unpublished data). This mutant failed to induce transcription in the mouse extract (Fig. 2). To test the importance of the proposed transactivation domain for *in vitro* transcription, we used a truncated protein prepared by cleavage of the template at a position corresponding to amino acid 122. This protein retains a functional DNA-binding domain but lacks sequences required for transcriptional activation *in vivo* (11). In the *in vitro* system, this ACE1-(1–122) peptide was a less effective activator than the full-length protein but, surprisingly, did give a certain level of Cu-dependent transcription (Fig. 2). Although activation was related to the concentration of ACE1-(1–122) peptide, saturation was obtained at a lower protein concentration than for the intact protein and the final extent of activation was lower by a factor of 4. In several repetitions of this experiment, the truncated protein activated transcription 20–35% as well as the wild-type protein. It is unknown whether the 11 amino acids of ACE1-(1–122) past

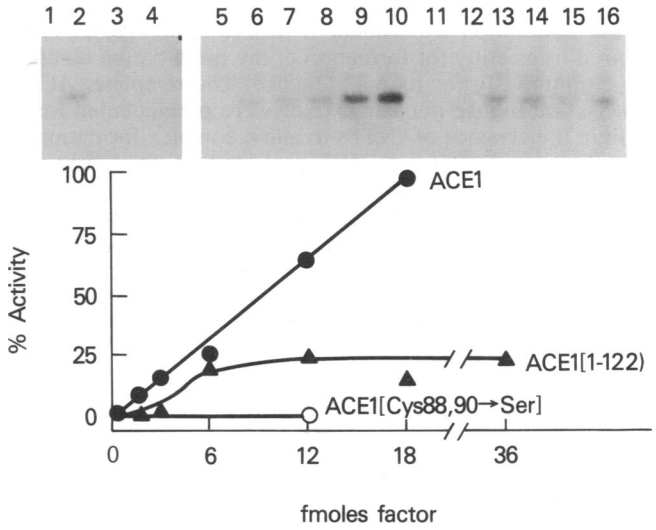


FIG. 2. Analysis of ACE1 mutants. (*Upper*) *In vitro* transcription of the CUP1:CAT construct was performed in the presence (lanes 2 and 4–16) or absence (lanes 1 and 3) of 25 μM Cu^{I}-acetonitrile in reaction mixtures containing 4 μl (lanes 1–4) or 6 μl of a wheat germ translation mixture containing the following amounts of ACE1 protein or mutant derivatives: 12 fmol of wild-type ACE1 (lanes 1 and 2); 12 fmol of mutant ACE1 in which Cys-88 and -90 in the full-length protein have been converted to serines (lanes 3 and 4); 0, 1.5, 3, 6, 12, or 18 fmol of wild-type ACE1 (lanes 5–10); 1.5, 3, 6, 12, 18, or 36 fmol of ACE1-(1–122) (lanes 11–16), a truncated ACE1 translated from a mRNA produced from a SP6–ACE1 template that had been restricted with *Bgl* II at the codon for amino acid 122 (11). (*Lower*) Transcripts were quantitated by densitometry and plotted as a percentage of the maximal activity obtained with the wild-type ACE1 protein. ●, wild-type ACE1; ○, $[Ser^{88,90}]$ACE1; ▲, ACE1-(1–122).

the postulated DNA-binding domain act as a "cryptic activator," as has been observed for a similar truncation mutant of the GAL4 regulatory protein (20) or whether DNA-binding by itself leads to a low level of transcriptional activation.

Metal Specificity. As an additional comparison of transcription *in vitro* and *in vivo*, we determined the ability of various metals to activate CUP1:CAT transcription in the presence of ACE1 protein (Table 2). The divalent cations Zn(II), Pb(II), Cd(II), and Ni(II) were all incapable of stimulating transcription at the concentrations tested. These ions also fail to induce *CUP1* gene transcription *in vivo* (6, 7). In contrast, Ag(I), which activates the ability of ACE1 to bind DNA *in vitro* and induces *CUP1* gene transcription *in vivo* (11), induced *in vitro* transcription as well as Cu(I) but required a slightly higher concentration of added metal. Ag(I) is isoelectronic with Cu(I) and binds to metallothionein with similar stoichiometry and geometry.

ACE1 Is Required for Maintenance of the Preinitiation Complex. Transcription involves at least three discrete steps: formation of a preinitiation complex (which involves formation of both a "template committed complex" and a "fast start complex"), initiation, and elongation (27). Recent studies on the ATF transcription factor have led to the proposal that eukaryotic transcriptional activator proteins may be needed only transiently for assembly but not for maintenance of the committed preinitiation complex (28, 29). To evaluate the generality of this hypothesis, we took advantage of the fact that since ACE1 depends on Cu for DNA binding, it can be completely dissociated from DNA by Cu-complexing agents (11).

In preliminary experiments, we found that both KCN and diethyldithiocarbamate completely inhibited transcription from ACE1-dependent promoters but not from ACE1-independent promoters, including the mouse metallothionein I, trout metallothionein B, and Rous sarcoma virus (RSV) promoters (data not shown). This allowed us to perform an order-of-addition experiment to test whether ACE1 is required transiently for formation of the preinitiation complex or continuously for its maintenance. The template, ACE1, Cu(I), and mouse nuclear extract were preincubated for 50 min in the absence of rNTPs to allow complex formation. A Cu chelator, CN^- (as KCN) was added either at time 0 or after 40 min, and transcription was started at 50 min (Fig. 3 *Lower*). KCN completely inhibited the Cu-dependent transcription of UASc:mTATA:CAT whether it was added at time 0 (Fig. 3, lane 2 vs. lane 1) or after 40 min of preincubation (lane 3 vs. lane 1). As a control for any possible nonspecific effects of KCN on general transcription factors or RNA polymerase, a similar experiment was performed on an RSV template. In this case, KCN had no effect on transcription (lane 3 vs. lane 4). To demonstrate that a 40-min preincubation is sufficient to form a committed preinitiation complex, we repeated the experiment of lanes 1–3 using 0.015% sarkosyl instead of KCN as the inhibitor; this concentration of sarkosyl inhibits the formation of both the "template committed complex" and the "fast start complex" (27). Sarkosyl completely inhibited transcription when added at the start of the reaction (lane 8) but had no effect when added after a 40-min preincubation (lane 9). This shows that 40 min is sufficient to form a committed complex, as defined by the usual criterion of resistance to sarkosyl, and further that there is no significant level of reinitiation in our transcription system. Additional experiments, in which three rNTPs were added to allow formation of an initiated complex, suggested that the main effect of ACE1 is on initiation rather than elongation (data not shown). These results indicate that ACE1 is required for the maintenance as well as the assembly of a functional preinitiation complex.

Table 2. Metal specificity of ACE1-mediated transcription

Metal salt	Concentration, μM	Relative transcription
None	—	<5
$Cu(CH_3CN)_2^+$	3	13
	12.5	75
	50	100
	200	15
$AgNO_3$	25	49
	150	89
	300	33
$CdSO_4$	5, 50, 500	<5
$ZnSO_4$	10, 50, 125	<5
$NiCl_2$	10, 50, 125	<5
$Pb(NO_3)_2$	10, 50, 125	<5

In vitro transcription mixtures containing the CUP1:CAT template DNA and 12 fmol of *in vitro* synthesized ACE1 were supplemented with various concentrations of the indicated metal salts. Transcriptional activity was quantitated as in Table 1 and values are presented as a percentage of the activity obtained with 50 μM Cu(I).

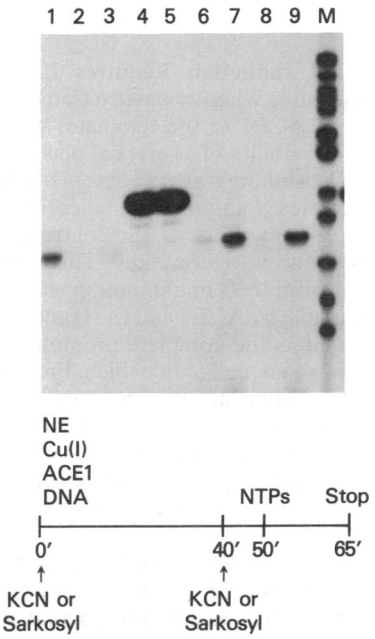


FIG. 3. Reversibility of Cu induction. *In vitro* transcription mixtures contained UASc:mTATA:CAT (lanes 1–3 and 6–9) or RSV:CAT (lanes 4 and 5) templates, 50 μM Cu^I-acetonitrile (lanes 1–5 and 7–9), and 16 fmol of *in vitro* translated ACE1 (all lanes). The mixtures were preincubated at 30°C for 50 min in the absence of rNTPs. As shown in the scheme below the autoradiogram, the mixtures, containing mouse nuclear extract (NE), were supplemented with 20 mM KCN at time 0 (lane 2) or 40 min (lanes 3 and 5) or with 0.015% sarkosyl at time 0 (lane 8) or 40 min (lane 9). At 50 min, transcription was initiated by the addition of rNTPs (500 μM each). The reactions were stopped at 65 min and the RNA was analyzed by primer extension. The 104-base reverse transcriptase product from the UASc:mTATA:CAT template represents 86 bases from the CAT gene and 18 bases from the mouse metallothionein I gene (22). The 125-base reverse transcriptase product from the RSV template represents 86 bases from the CAT gene and 39 bases from RSV (30). Lane M, pBR322 digested with *Msp* I to provide size markers.

DISCUSSION

The results show that the *in vitro* transcription system faithfully recreates *in vivo* metallothionein gene transcription in terms of template, activator protein, and metal specificity. The ability of the yeast regulatory protein to function together with mouse general transcription factors provides

further evidence for the remarkable functional conservation of the eukaryotic transcriptional apparatus (16–20). Although a large number of yeast transcriptional regulatory proteins have now been cloned and analyzed genetically, cell-free transcription systems from yeast are not as well characterized as those from mammalian cells. The ability to use a yeast factor in a mammalian extract provides a convenient method to study the biochemistry of gene regulation.

Our data support the idea that ACE1, like several other eukaryotic transcription factors (13–15), is a two-domain protein with distinct DNA-binding and activation functions. We previously showed (11) that a carboxyl-terminal deletion mutant of ACE1 was not able to transactivate *CUP1* gene expression *in vivo*; however, the interpretation of this experiment was ambiguous since this mutation could affect protein stability as well as function. In the *in vitro* system, we can clearly show that the truncated protein is present and binds to DNA yet activates transcription less well than the full-length protein. We also show here that transcriptional activation requires the presence of at least two cysteine residues, the postulated ligands for Cu. This supports our hypothesis that binding of Cu to cysteine residues folds the protein into a conformation that can bind DNA and thus activate transcription (11).

Two observations show that the mouse general factors prefer to use mouse as compared to yeast downstream regulatory sequences. (*i*) A construct containing a mouse TATA and initiation site was transcribed about 6 times better than an equivalent construct containing yeast TATA and initiation sequences (Table 1, compare UASc:mTATA:CAT with UASc:yTATA:CAT). (*ii*) When the mouse extract was presented with a purely yeast promoter, it exclusively used a mouse-like initiation site rather than the normal yeast site (Fig. 1). This preference was not observed in previous cross-species experiments, because the fusion genes all contained downstream sequences derived from the host species (16–20). These results suggest that general promoter factors, like specific activator proteins, can be thought of as consisting of at least two functionally distinguishable domains: a DNA-recognition domain, which in the case of mouse factors prefers mouselike sequences, and an activation domain, which can cooperate with proteins from diverse species.

A special feature of the ACE1 system is that transcription can be specifically inhibited by Cu-complexing agents such as CN^- or diethyldithiocarbamate. This allowed us to perform an order-of-addition experiment to determine whether ACE1 is required transiently or continuously for formation of a functional initiation complex. Our results show that ACE1 is required for transcription even after the formation of a committed preinitiation complex (as defined by resistance to sarkosyl). Control experiments using several ACE1-independent templates show that the effect of these chelators is specific for ACE1 and is not an artifact due to inhibition of general transcription factors or RNA polymerase. Although the precise step(s) for which ACE1 is required is not known, preliminary experiments indicate that it occurs before elongation. Our results differ from those recently obtained with the ATF regulatory factor and an adenovirus template (28, 29). Based on experiments in which an oligonucleotide was used to mediate dissociation of the regulatory factor from the template, it was concluded that ATF may be required only transiently for the formation of a committed complex. However, it is difficult to interpret this experiment because the dissociation of ATF was followed by DNase footprinting, which requires a majority of the templates to be occupied, whereas only a minority of templates are actually involved in transcription; thus it is possible that ATF was still present on the active templates. By using a chemical inhibitor, KCN, it was possible to completely remove the ACE1 regulatory factor from the template as shown both by the complete inhibition of transcription (Fig. 3) and by electrophoretic mobility-shift assays (data not shown). An alternative possibility is that ACE1 activates transcription by a different mechanism or through different auxiliary factors than ATF. The ability to reversibly activate metallothionein gene transcription *in vitro* should allow further insights into the biochemical mechanism by which a specific regulatory protein activates eukaryotic gene transcription.

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