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A Specialized Nucleosome Modulates Transcription Factor Access to a C. glabrata Metal Responsive Promoter

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Summary

The ability of DNA binding transcription factors to access *cis*-acting promoter elements is critical for transcriptional responses. We demonstrate that rapid transcriptional autoactivation by the Amt1 Cu metalloregulatory transcription factor from the opportunistic pathogenic yeast Candida glabrata is dependent on rapid metal-induced DNA binding to a single metal response element (MRE). In vivo footprinting and chromatin-mapping experiments demonstrate that the MRE and a homopolymeric (dA • dT) element adjacent to the MRE are packaged into a positioned nucleosome that exhibits homopolymeric (dA • dT)-dependent localized distortion. This distortion is critical for rapid Amt1 binding to the MRE, for Cu-dependent *AMT1* gene transcription, and for C. glabrata cells to mount a rapid transcriptional response to Cu for normal metal detoxification. The *AMT1* promoter represents a novel class of specialized nucleosomal structures that links rapid transcriptional responses to the biology of metal homeostasis.

Introduction

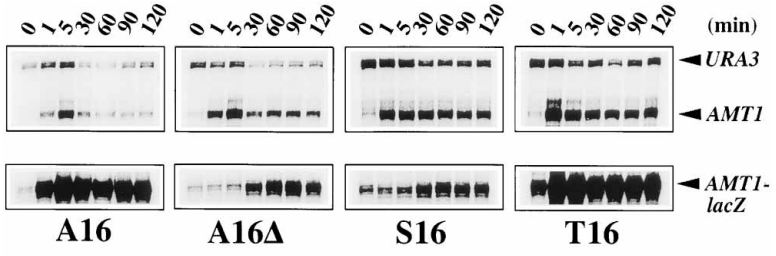
Rapid transcriptional responses play a key role in organismal development, in adaptations to physiological signals, and in response to stresses such as heat, metals, and free radicals. In many cases, these induced transcriptional responses involve the signal-dependent sequence-specific DNA binding by *trans*-activator proteins that stimulate transcription. A number of inducible eukaryotic DNA binding *trans*-activators have been studied, including NF-κB in response to infection and inflammation, Heat Shock Factor 1 in response to thermal stress, and the ligand-dependent activation of hormone receptors (Grimm and Baeuerle, 1993; Tsai and O'Malley, 1994; Wu, 1995). Therefore, one rate-limiting step in inducible gene transcription activation is determined by the kinetics of *trans*-activator interactions with their cognate DNA binding sites in promoters.

The packaging of DNA in vivo into chromatin has been well established both to augment specific gene activation by enhancing interactions between distally DNA-bound proteins and to repress gene expression by limiting access of DNA binding proteins to *cis*-acting regulatory elements (Felsenfeld, 1992; Lewin, 1994; Paranjape et al., 1994; Kornberg and Lorch, 1995; Kingston et al., 1996; Struhl, 1996). A number of studies have demonstrated that chromatin structure may provide a preset architecture for transcription-factor access by positioning nucleosomes in such a way that *cis*-acting sites are nonnucleosomal. This is accomplished through the use of promoter DNA binding proteins such as GAGA factor or of components of the transcriptional machinery such as TFIID or RNA Polymerase II (Hayes and Wolffe, 1992; Wallrath et al., 1994; Shopland et al., 1995). A hallmark of preset promoters is the presence of constitutive DNase I hypersensitive sites encompassing *cis*-acting regulatory elements, which reflect the accessibility of such regions to DNA binding proteins (Wu, 1980; Gross and Garrard, 1988).

In contrast to preset promoters, many *cis*-acting promoter regulatory elements are packaged into nucleosomes that, in response to the activation signal, are remodeled to allow facile access by *trans*-activators (Wallrath et al., 1994). The appearance of inducible DNase I hypersensitive sites under conditions of gene activation is diagnostic of chromatin-remodeling events (Wallrath et al., 1994). A number of factors have been demonstrated to be involved in nucleosome disruption and remodeling, including the binding of transcription factors themselves, histone acetylation or other post-translational modifications, and the use of ATP-dependent protein complexes such as SWI/SNF from yeast or NURF from Drosophila (Fascher et al., 1990; Peterson and Tamkun, 1995; Shopland et al., 1995; Tsukiyama and Wu, 1995; Wilson et al., 1996; Wolffe and Pruss, 1996).

A striking example of rapidly induced transcriptional responses is observed for the toxic metal–responsive metalloregulatory transcription factor Amt1 in the opportunistic pathogenic yeast Candida glabrata (Zhu et al., 1995). When C. glabrata cells are exposed to elevated Cu levels, Amt1 coordinates Cu(I) ions via the amino-terminal DNA binding domain. Cu binding to Amt1 drives a conformational change that activates sequence-specific binding to metal response elements (MREs) in the promoters of genes encoding the metallothionein (MT) metal detoxification proteins. Once bound to multiple MREs in the promoters of the *MT-I*, *MT-IIa*, and *MT-IIb* isoform genes, the carboxy-terminal *trans*-activation domain of Amt1 potently activates transcription, resulting in protection from toxic levels of Cu (Zhou et al., 1992). Although Amt1-dependent MT gene transcription occurs very quickly, the initial response to Cu is rapid Amt1 transcriptional autoactivation (Zhou and Thiele, 1993). *AMT1* autoactivation occurs via the Cu-dependent binding of Amt1 to a single MRE, which is essential for C. glabrata cells to provide sufficient transcription factor for the subsequent activation of the MT gene family (Zhou and Thiele, 1993). In vitro Amt1 binds to this MRE as a monomer and makes contacts in both the major and minor groove that are critical for DNA binding in vitro and transcriptional activation in vivo (Koch and Thiele, 1996).

Here, we demonstrate that a homopolymeric (dA • dT) element, located adjacent to the *AMT1* MRE, plays a critical role in rapid transcriptional autoactivation of the *AMT1* gene. Although the *AMT1* promoter region encompassing the homopolymeric (dA • dT) element and the MRE is packaged within a positioned nucleosome in vivo, the homopolymeric (dA • dT) element confers



AMT1-lacZ Promoter Derivative	Fold Induction 5 min	60 min
A16 lacZ	11	19
A16Δ lacZ	1	11
S16 lacZ	1	8
T16 lacZ	15	23

Figure 1. The Homopolymeric (dA • dT) Element Is Required for Rapid *AMT1* Cu-Responsive Transcriptional Autoactivation

(Top) RNase protection assay of the kinetics of Cu-dependent transcriptional activation of the wild-type (A16) and mutant (A16Δ, S16, and T16) *AMT1-lacZ* promoter derivatives. Wild-type cells containing the indicated promoter derivative were treated with 100 μM $CuSO_4$ for the indicated times, harvested, and 10 μg of total RNA used in RNase protection assays. The RNase protection productions were subjected to electrophoresis on a 6% polyacrylamide-urea gel. The AMT1-lacZ, endogenous AMT1, and URA3 RNase protection products are indicated with arrowheads as *AMT1-lacZ*, *AMT1*, and *URA3*.

(Bottom) Quantitation of fold induction at 5 or 60 min for the *AMT1-lacZ* promoter derivatives from the data shown in the top panel.

a localized deformation of the nucleosomal DNA. This nucleosomal structure fosters rapid Cu-activated Amt1 DNA binding and *AMT1* transcription in vivo and is essential for C. glabrata cells to mount a rapid transcriptional response to Cu for normal metal detoxification and cell division. These studies suggest that specialized nucleosomes, via DNA elements that modulate intranucleosomal structure, provide a mechanism for fostering rapid transcription factor access to chromatin to evoke immediate responses to extracellular stimuli.

Results

Rapid *AMT1* Transcription Requires a Homopolymeric (dA • dT) Element

Previous investigations demonstrated that the C. glabrata *AMT1* gene is rapidly autoactivated via a single *AMT1* promoter MRE (Zhou and Thiele, 1993). Five nucleotides upstream of this MRE lies a homopolymeric (dA • dT) element, denoted A16, that neither plays a role in the high affinity Cu-Amt1 binding in vitro nor confers detectable *AMT1* promoter distortion (Koch and Thiele, 1996). A number of reports have identified homopolymeric (dA • dT) stretches in promoter regulatory regions. In the yeast *HIS3* promoter, a poly (dA • dT) sequence has been shown to play an important role in Gcn4p-mediated transcription by increasing the accessibility of Gnc4p to its cognate DNA binding site (Iyer and Struhl, 1995). To ascertain whether the A16 element plays a role in *AMT1* transcriptional autoactivation, we compared the kinetics of Cu-activated transcription from the wild-type *AMT1-lacZ* fusion plasmid, pAMT1-lacZ, to derivatives in which the A16 element was mutationally altered. We previously demonstrated that this *AMT1-lacZ* fusion plasmid is regulated by Cu in a manner that is indistinguishable from the endogenous *AMT1* gene (Zhou and Thiele, 1993). Furthermore, a number of studies in both S. cerevisiae and S. pombe have demonstrated that the chromatin structure of genes carried on episomal plasmids is indistinguishable from that of the endogenous chromosomal loci (Perez-Ortin et al., 1987; Bernardi et al., 1991). Figure 1 demonstrates that, as previously observed, the wild-type *AMT1-lacZ* fusion plasmid (A16) is rapidly transcriptionally activated in response to Cu. However, either deletion of the A16 element (A16Δ) or scrambling of this element into random sequence predicted to assume B-form DNA structure (S16) severely reduced the kinetics of Cu-activated *AMT1-lacZ* transcription (Figure 1, upper panel). A mutant in which the poly (dA • dT) sequence was retained but was flipped to the opposite strand (T16) displayed the same rapid Cu-activated transcription as the wild-type promoter. Quantitation of mRNA levels showed that after a 5 min exposure to Cu, the wild-type and T16 promoter fusions were induced 11- and 15-fold, respectively (Figure 1, lower panel). While the A16Δ and S16 promoter fusions were not activated at this assay time, the endogenous chromosomal *AMT1* gene was rapidly transcriptionally activated for each of the four strains assayed (Figure 1, upper and lower panels). After a 60 min incubation in the presence of Cu, strains harboring the A16 and T16 promoters expressed *AMT1-lacZ* fusion mRNA levels that were elevated 19- and 23-fold, respectively, over basal levels. Interestingly, the A16Δ and S16 promoter derivatives were induced 11- and 8-fold, respectively, the late activation of which is dependent on both Cu and a wild-type endogenous *AMT1* gene (data not shown). These results clearly demonstrate that the A16 element plays an important role in fostering rapid Cu-inducible *AMT1* gene transcription.

The A16 Element Fosters Rapid Inducible Amt1 Binding In Vivo

We previously demonstrated that purified Amt1 binds to a wild-type or A16Δ promoter DNA fragment in vitro with indistinguishable affinities ($K_d = 2 - 3 \times 10^{-10}$ M; Koch and Thiele, 1996). However, because the A16 element is important for the rapid kinetics of *AMT1* transcription in vivo, we analyzed the kinetics of Cu-induced Amt1 binding to the MRE in plasmids containing the wild-type *AMT1* allele or the S16 mutant allele in C. glabrata cells by in vivo dimethyl sulfate (DMS) footprinting. The data in Figure 2A demonstrate that two G

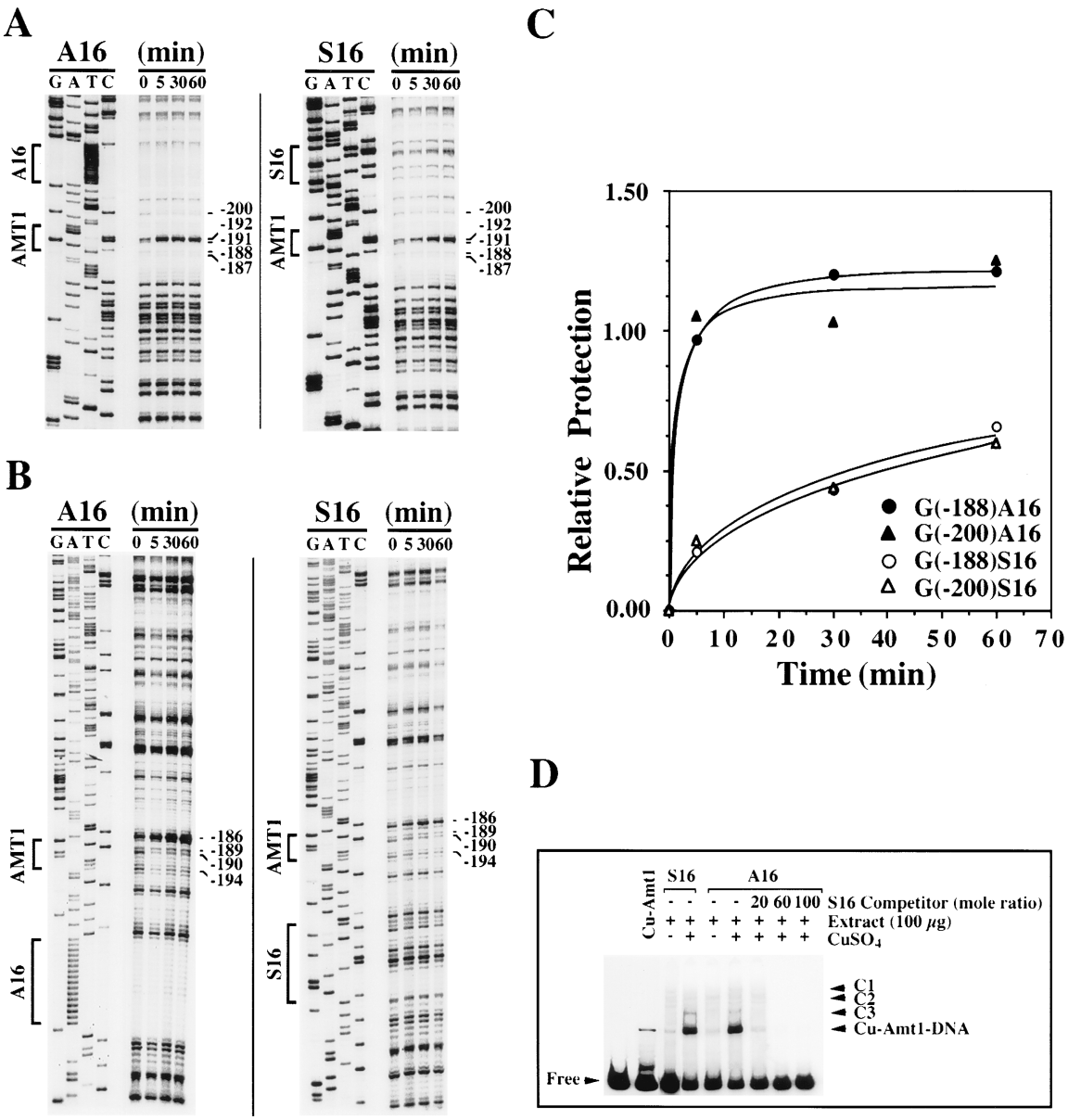


Figure 2. Kinetics of Amt1 Binding In Vivo Detected by DMS Footprinting of the Wild-Type (A16) and the Mutant (S16) *AMT1* Promoters

C. glabrata cultures containing either the wild-type or S16 *AMT1* promoter mutation were incubated in the absence (0) or presence of 100 μM $CuSO_4$ for 5, 30, or 60 min followed by a 5 min incubation with dimethyl sulfate. DNA was isolated and the sites of modification were detected by cleavage and ^{32}P-labeled oligonucleotide primer extension reactions using primer p6 (panel A, coding strand) and p4 (panel B, noncoding strand). The primer extension products were fractionated on a 6% polyacrylamide-urea gel. AMT1, the MRE; A16, the homopolymeric (dA • dT) stretch in the wild-type *AMT1* promoter; S16, the scrambled sequence in the mutant promoter. The reference DNA sequencing reactions were performed using the same primers with plasmids pRSAMT1 and pRSS16, respectively. (C) Quantitation of the kinetics of methylation protection at the major groove G residues −188 and −200 in the Amt1 binding site. The protection was normalized using a reference cleavage site distal to the Amt1 binding site. (D) Electrophoretic mobility shift assays using ^{32}P-labeled *AMT1* promoter fragments derived from the wild-type (A16) or mutant (S16) promoters. Whole cell extracts (100 μg) from either control cells (−) or 100 μM $CuSO_4$ induced cells (+), or Cu(I) saturated Amt1p purified from E. coli, were used in binding reactions in the presence of either the indicated molar excess or unlabeled S16 promoter fragment. Free represents free probe DNA; Cu-Amt1-DNA, the Cu-Amt1p-DNA complex; and C1, C2, and C3, additional *AMT1* promoter DNA binding activities.

residues within the MRE on the coding strand, −188 and −200, are protected from DMS methylation within 5 min after Cu exposure. In contrast, these same residues are poorly protected, even after 60 min Cu exposure, in the S16 allele (Figure 2A). Furthermore, we observed DMS hypersensitivity at G −192, which was maximal for the wild-type allele at the 5 min time point; however, this residue was maximally modified in the S16 promoter ~30 min after Cu exposure. Quantitation of the methylation protection for G residues −188 and −200 is shown in Figure 2C. On the noncoding strand, the wild-type *AMT1* promoter was rapidly protected from DMS

methylation at positions −193, −194, and −195 (Figure 2B), adenosine residues that are known by in vitro DNA binding experiments to make important minor groove contacts with Amt1 (Koch and Thiele, 1996). Little if any protection of these residues was observed at any time for the S16 promoter. Additionally, in the wild-type promoter, the G −190 residue is rapidly protected from DMS and the G −186 is rapidly hypermethylated in response to Cu. However, the protection and hyperreactivity of these same residues in the S16 promoter occurs to only a limited extent and with much slower kinetics (Figure 2B). The in vivo DMS footprinting demonstrates that the A16 element is required for rapid Amt1 binding. Moreover, the kinetics of the MRE binding by Cu-Amt1 parallel the observed kinetics of gene transcription in vivo.

The importance of the A16 element for rapid Cu-activated Amt1 binding to the MRE in vivo, but not to naked DNA in vitro, suggests that the A16 stretch functions to recruit Amt1 to the MRE in vivo. This might be accomplished via two mechanisms. The A16 element may be recognized by a C. glabrata DNA binding protein that may foster Amt1 binding either via protein–protein interactions or by inducing conformational changes in chromatin to allow greater Amt1 access. Alternatively, the A16 element might, by virtue of the structural rigidity of homopolymeric (dA • dT) sequences (Nelson et al., 1987), assume a structure in chromatin that provides greater access of Cu-Amt1 to the adjacent MRE. To address these possibilities, we conducted in vitro electrophoretic mobility shift experiments using whole cell extracts from wild-type C. glabrata cells, either untreated or treated with 100 μM $CuSO_4$. The data in Figure 2D demonstrate that a Cu-Amt1-DNA complex (Cu-Amt1-DNA), as well as low levels of three additional complexes (C1, C2, and C3), is formed using either the wild-type (A16) or S16 *AMT1* promoter fragments. Furthermore, all of the complexes formed on the A16 promoter fragment were competed using the S16 DNA fragment as competitor. These data and in vivo DMS footprinting data (Figure 2) strongly suggest that there is no DNA binding activity in C. glabrata cells that binds to the A16 element in the *AMT1* promoter.

The A16 Element and MRE Are Embedded within a Micrococcal Nuclease Resistant Structure

To determine whether the A16 element modulates chromatin structure in the *AMT1* promoter, we subjected chromatin in permeabilized C. glabrata cells containing either the wild-type or S16 *AMT1* promoter mutation to limited micrococcal nuclease (MNase) digestion. Since it has been demonstrated that MNase exhibits a significant degree of sequence specificity for DNA cleavage, which may result in misinterpretation of chromatin analyses (McGhee and Felsenfeld, 1983), we compared the MNase digestion products from C. glabrata chromatin to those obtained using protein-free DNA by primer extension analysis. The data in Figure 3A demonstrate that for comparable degrees of MNase digestion in both naked and chromatin DNAs, the digestion patterns are distinct. In chromatin, both the A16 and S16 *AMT1* promoters are highly protected from MNase digestion

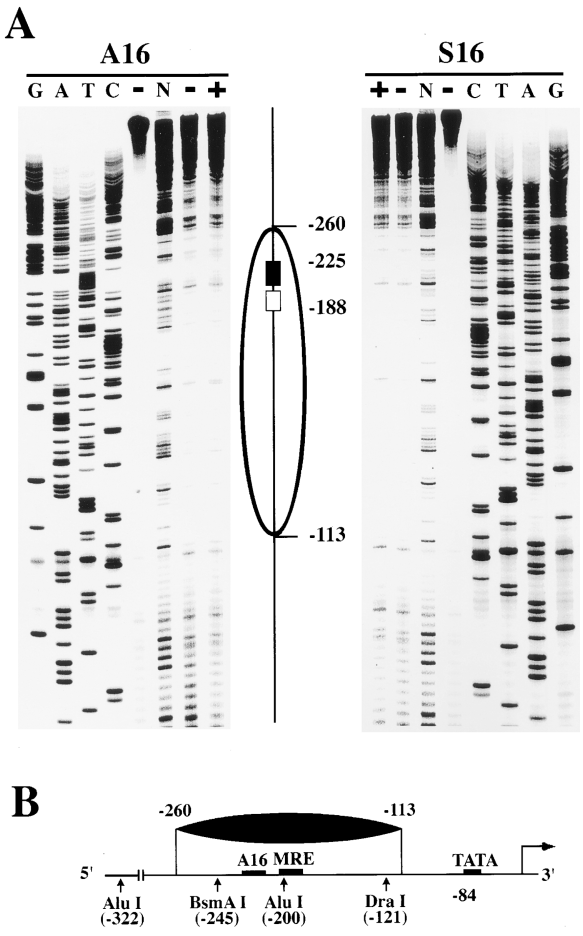


Figure 3. The *AMT1* Homopolymeric (dA • dT) Element and MRE Are Contained within a Nucleosome In Vivo

(A) C. glabrata cultures containing either the wild-type or S16 *AMT1* promoter mutation were incubated in the absence (−) or presence (+) of 100 μM $CuSO_4$ for 10 min, converted to spheroplasts, permeabilized with NP-40, and digested with MNase. DNA was used in primer extension footprinting reactions and the reaction products fractionated on an 8% polyacrylamide urea gel. Lanes: −, naked uncleaved DNA; N, naked DNA cleaved partially with MNase in vitro; (−), DNA from control cells treated with MNase; and (+), DNA from Cu-induced cells treated with MNase. The positions in the *AMT1* promoter giving the borders of MNase resistance are shown; the closed rectangle represents the A16 element, the open rectangle the metal response element (MRE), and the open ellipse the position of the nucleosome.

(B) Summary of the position of the nucleosome in the *AMT1* promoter. The positions of the A16 element, MRE, TATA box, and start site for *AMT1* gene transcription are shown. The positions of the AluI and Bsm AI sites are shown.

within the region extending from approximately −113 to −260 on the coding strand. Within this region there are a number of MNase cleavages, but their intensities are markedly reduced as compared to the same region on the naked DNA templates. These distinctions strongly suggest that this region of both the wild-type (A16) and S16 mutant promoters is present in cells as a nucleo-protein complex (Noll and Kornberg, 1977). No marked difference in the cleavage pattern was observed between the wild-type or S16 mutant *AMT1* promoter

either from control cells or Cu-induced cells. Independent primer extension experiments using purified mononucleosomal DNA gave rise to similar boundaries of MNase protection (data not shown). This region of ~147 nucleotides closely correlates with the highly conserved length of DNA wrapped into a nucleosomal core particle (Noll and Kornberg, 1977). Therefore, the results described here, and the ~10 bp periodicity of DNase I cleavage within this region (see below), suggest that the *AMT1* promoter, encompassing the A16 (or S16) element and the MRE, is packaged into a stably positioned nucleosome (Figure 3B).

A16-Mediated Localized Nucleosomal Distortion in the *AMT1* Promoter

A number of experiments have demonstrated that sites of nuclease hypersensitivity are indicative of accessible *cis*-acting regulatory elements (Wu, 1980; Gross and Garrard, 1988). The lack of evidence for an A16-specific DNA binding activity, coupled with the indication that the *AMT1* promoter A16 element and MRE are nucleosomal and that the A16 element is required for rapid access to the chromatin template by Amt1 in vivo, suggests that A16 may confer a chromatin structure that allows rapid Amt1 binding and gene transcription. To investigate the chromatin structure in the wild-type and S16 mutant promoters in detail, in vivo DNase I footprinting was carried out in permeabilized spheroplasts. The DNase I cleavage pattern on both strands of the *AMT1* promoter was assessed by primer extension reactions with deproteinized chromatin DNA and with naked DNA partially digested with DNase I in vitro. As shown in Figure 4A, treatment of cells containing the wild-type *AMT1* promoter with Cu for only 10 min resulted in the protection of several nucleotide residues on the coding strand (−186, −190, −191, −195, −198, −199, and −205) that correspond to residues within the Amt1 MRE (indicated with a closed circle) (Koch and Thiele, 1996). In contrast, the coding strand from the S16 promoter showed no residues protected from DNase I cleavage in response to Cu treatment for 10 min, consistent with the poor Amt1 binding (Figure 2) and transcriptional activation (Figure 1) we observed at this time following Cu administration. On the noncoding strand of the wild-type *AMT1* promoter, position −189 in the MRE was only slightly protected from DNase I digestion by Cu-Amt1 binding, but no protection was observed at this position for the S16 promoter (Figure 4B). These data are consistent with the DMS footprinting (Figure 2), which demonstrated the requirement for the A16 element for rapid Cu-induced binding of Amt1 to the MRE in vivo.

MNase digestion data presented in Figure 3 suggest that the A16 element and the MRE within the *AMT1* promoter may be packaged within a nucleosome. A characteristic feature of stably positioned nucleosomes is the occurrence of ~10 bp periodicity of DNase I cleavage (Simpson and Stafford, 1983). The data shown in Figures 4A and 4B (and summarized by phosphorImager quantitation in Figure 4C) demonstrate that DNase I cleavage occurs with ~10 to 11 bp periodicity for both the A16 and S16 *AMT1* promoters in chromatin. This is apparent on both the coding and noncoding strands and extends from approximately −126 to −261, beyond which this periodicity discontinues within the vicinity of the *AMT1* promoter. This region of ~10 bp DNase I cleavage periodicity is almost colinear with the region of the *AMT1* promoter protected from MNase digestion as shown in Figure 3. In contrast, analysis of a comparable extent of digestion of naked DNA with DNase I in vitro shows a distinct pattern of cleavage as compared to chromatin and lacks the ~10 bp periodicity. Furthermore, as observed for the protection of this region in chromatin from micrococcal nuclease digestion (Figure 3), the ~10 bp periodicity of DNase I cleavage does not change upon treatment of cells with Cu. Together, these data strongly suggest the presence of a stably positioned nucleosome within the *AMT1* promoter that encompasses the A16 (or S16) element and the MRE. Moreover, this nucleosome is not disrupted upon Cu-Amt1 binding and gene transcription.

In addition to the Cu-dependent DNase I protection of the MRE in the wild-type *AMT1* promoter, we observed pronounced DNase I hypersensitivity (DH) at several nucleotide positions on the coding strand (−228, −229, −236, and −237) and the noncoding strand (−203 and −206), which are found immediately upstream and downstream, respectively, of the A16 element (DH, Figures 4A–4C). These sites in the A16 promoter are 10-fold hypersensitive to DNase I cleavage with respect to the corresponding sites in the S16 promoter derivative, as ascertained by quantitative phosphorImaging. DNase I cleavage at these residues occurs under noninduced conditions, when Amt1 is not bound to the *AMT1* MRE and the degree of DNase I sensitivity at these positions does not change detectably upon Amt1 binding and gene activation. Furthermore, the DNase I hypersensitive sites downstream of the A16 element partially overlap with the *AMT1* MRE (Figure 4C). In contrast, no DNase I hypersensitivity is found at these positions in the S16 promoter (Figure 4). These observations suggest that this region of the S16 *AMT1* promoter derivative is significantly less accessible to DNase I in vivo than is the wild-type promoter. These data, combined with the demonstration that this region of the *AMT1* promoter is nucleosomal, suggest that prior to induction of Amt1 binding, the homopolymeric (dA • dT) element confers localized DNA distortion, which renders flanking sequences and the *AMT1* MRE hypersensitive to DNase I cleavage.

The data in Figures 4B and 4C also show that in the wild-type *AMT1* promoter, but not S16, there are DNase I cleavages that have been mapped with longer electrophoretic runs to nucleotide positions −64, −74, and −84 (indicated with brackets, TATA). Cleavage at these sites is attenuated in the wild-type *AMT1* promoter upon Cu addition and this protection occurs concomitantly with Amt1 binding to the MRE, but these sites are not protected from DNase I cleavage in the S16 promoter. Interestingly, a putative TATA box element is located within this region at positions −79 to −84, suggesting that, upon binding, Amt1 may interact with transcription components encompassing the TATA box either to induce binding or to stimulate conformational changes in prebound components. Furthermore, Cu-dependent enhanced cleavage by DNase I in the wild-type but not

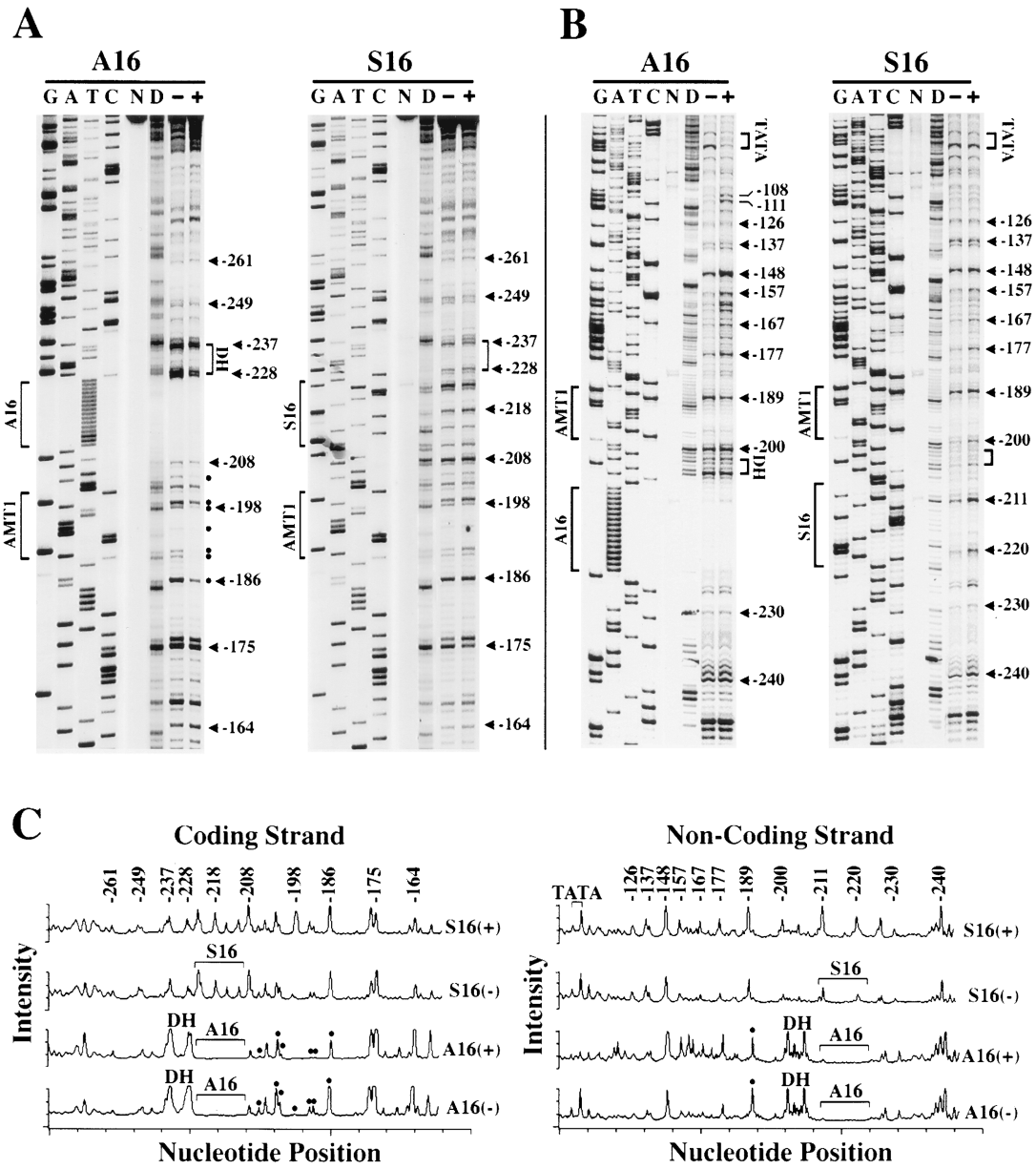


Figure 4. In Vivo DNase I Footprinting of the Wild-Type (A16) and Mutant (S16) *AMT1* Promoters

C. glabrata cultures containing either the wild-type (A16) or S16 *AMT1* promoter mutation were incubated in the absence (−) or presence (+) of 100 μM $CuSO_4$ for 10 min and subjected to in vivo DNase I footprinting as described in Experimental Procedures. (A) Coding strand and (B) noncoding strand. Lane (N) represents naked undigested DNA, lane (D) contains naked DNA digested in vitro with DNase I, and (−) and (+) indicate DNA extracted from cells untreated or treated with 100 μM $CuSO_4$, respectively. The bracket (AMT1) and bar from −186 to −205 indicates the position of the Amt1 binding site. The A16 and S16 elements are indicated with brackets. DNase I hypersensitive sites are bracketed (DH). Lanes G, A, T, and C represent DNA sequencing lanes as described in the legend to Figure 2. (C) Quantitative phosphorImaging of in vivo DNase I footprinting (A and B). The locations of the ~10 bp period DNase I cleavages on both strands for the wild-type and S16 promoters are indicated above each scan. The locations of the A16 or S16 elements are indicated with brackets. The MRE residues protected from DNase I cleavage in Cu-treated cells are indicated with closed circles. The DNase I hypersensitive sites flanking the A16 element, but absent in the S16 promoter, are indicated with DH. The location of DNase I cleavages encompassing the TATA box is indicated on the lower scan for the noncoding strand with brackets. The area within each peak, rather than the peak height, represents the relative cleavage frequency.

the S16 promoter is observed at positions −108 and −111, which map near the end of the nucleosomal core, within which lie the A16 and MRE elements (Figures 4B and 4C). The significance of these observations with respect to the mechanism of Cu-responsive *AMT1* gene transcription is currently under investigation.

The DNase I hypersensitivity of the wild-type but not the S16 *AMT1* promoter derivative suggests that the nucleosomal DNA immediately flanking the A16 element is locally accessible. To independently measure the relative accessibility of the wild-type and S16 *AMT1* promoters to DNA binding proteins, we used a modification of

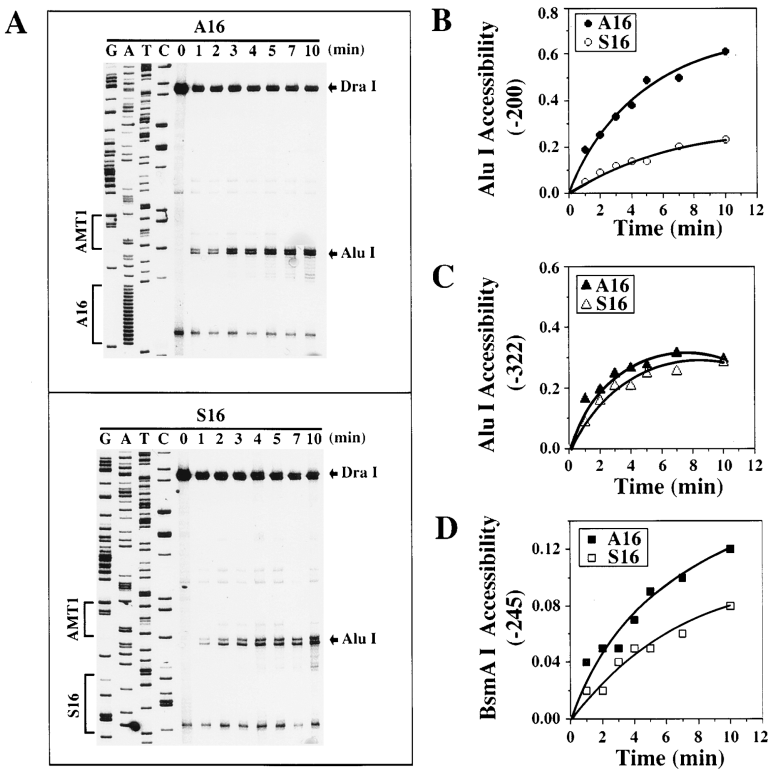


Figure 5. The Nucleosomal Homopolymeric (dA • dT) Element Augments Accessibility to Flanking Restriction Endonuclease Sites In Vivo

C. glabrata cultures containing either the wild-type (A16) or S16 AMT1 promoter mutation were grown in the absence of exogenous Cu, converted to spheroplasts. NP-40–permeabilized spheroplasts were incubated with AluI (50 U/ml) or Bsm A I (65 U/ml) for the indicated times. The cleavage rate at the nucleosomal AluI site (−200) and Bsm AI site (−245) was quantitated by oligonucleotide primer extension reactions (shown for AluI in panel A, A16 on top panel and S16 on lower panel). The G, A, T, and C lanes are DNA sequencing reactions. AluI accessibility was plotted as a function of the ratio of cleavages at sites AluI (−200) and DraI (−120) versus digestion time (B). The DraI cleavage reaction was taken to completion by digestion of the purified DNA in vitro. (C) represents the accessibility data plotted for the AluI (−322) site, which is outside of this nucleosome. (D) represents the accessibility data plotted for the Bsm AI (−245) site.

a method applied by Polach and Widom (1995) in which restriction endonucleases are used as probes of DNA accessibility within the nucleosome. C. glabrata cells harboring either the wild-type or S16 mutant *AMT1* promoter were grown in the absence of exogenous Cu, converted to spheroplasts, permeabilized with NP-40, and incubated with the restriction enzyme AluI, a recognition site for which is located at position −200 and lies within the *AMT1* MRE (Figure 3B). The AluI digestion products were detected by primer extension reactions with a ^{32}P-labeled oligonucleotide primer P4 and quantitated by phosphorImaging. The data in Figure 5 clearly demonstrate that over a time course of between 0 and 10 min, the rate of AluI cleavage in the wild-type promoter (A16) was much faster than the rate of AluI cleavage in the S16 promoter (Figure 5B), although the rates of cleavage of an upstream AluI site (detected with primer P10) in the *AMT1* promoter (position −322, Figure 3B) outside of the mapped A16-MRE nucleosome boundaries were comparable in both strains (Figure 5C). Cleavage of chromatin (detected with primer P2) with endonuclease Bsm AI, a site for which is located 20 nucleotides upstream of the A16 tract at position −245 (Figure 3B), gave faster cleavage kinetics for the wild-type *AMT1* promoter than for S16, consistent with the differences in cleavage rates observed for AluI (Figure 5D). Therefore, in agreement with the DNase I sensitivity at nucleotide positions flanking the A16 tract, but not the S16, restriction endonuclease cleavage rates strongly suggest that the A16 element confers a localized region of access to DNA binding proteins within the nucleosome mapped to the *AMT1* promoter region encompassing the MRE. Based upon the in vivo DNA binding experiments, DNase I sensitivity, and restriction endonuclease cleavage analyses, the DNA in the S16 promoter appears to follow a more regular path around the histone core of the nucleosome and is therefore less accessible to the DNA binding proteins DNase I, restriction endonucleases, and Cu-Amt1.

The Homopolymeric (dA • dT) Element Is Critical for the Response to Toxic Metals

Previous experiments have demonstrated that cells harboring a mutated MRE, to which Cu-Amt1 binds with a much lower affinity in vitro, are largely defective in *AMT1* transcriptional autoactivation and partially defective in MT gene activation and Cu detoxification (Zhou and Thiele, 1993). Since Cu is an essential metal that is also highly cytotoxic, we tested whether cells that harbor an *AMT1* gene with a functional MRE but that display dramatically slower activation kinetics and altered nucleosomal structure due to mutagenesis of the A16 element are defective in Cu detoxification. The wild-type (A16) and S16 alleles of the *AMT1* were integrated at the *ura3*$^-$ locus in single copy in a strain in which the endogenous *AMT1* gene had been insertionally inactivated. A time course of exposure to 10 μM Cu demonstrated that the wild-type strain, *A16::URA3*, exhibited the rapid kinetics of *AMT1* gene transcriptional activation observed with both the endogenous wild-type *AMT1* gene and the plasmid-borne *AMT1-lacZ* fusion gene (Figure 6A). However, the *S16::URA3* strain displayed delayed activation of *AMT1* mRNA expression by Cu similar to that observed for the *S16-lacZ* fusion gene (Figure 6A and Figure 1). The magnitude of activation of the *AMT1* promoter in these experiments is lower

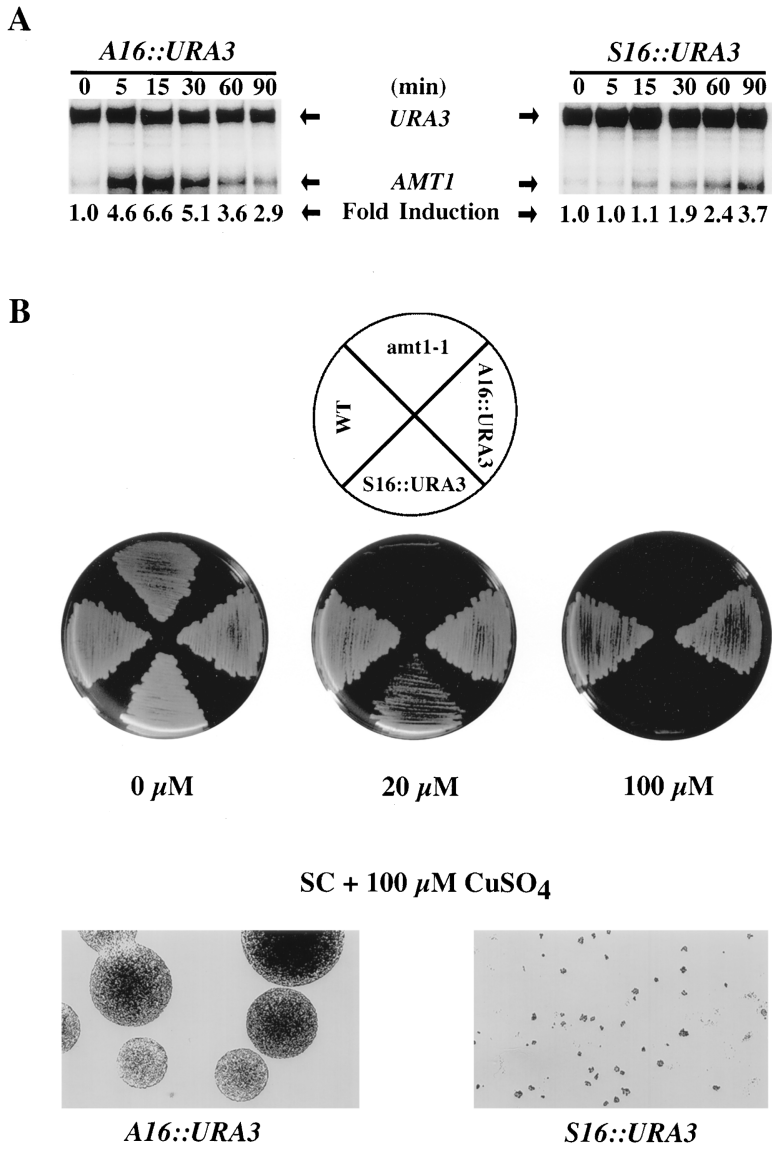


Figure 6. The Nucleosomal Homopolymeric (dA • dT) Element Is Essential to Mount a Rapid Cu Detoxification Response

(A) The wild-type and S16 *AMT1* alleles integrated at *ura3*. C. glabrata *amt1–1* cells harboring the wild-type or S16 *AMT1* allele, integrated at *ura3*, were assayed by RNase protection experiments for the kinetics of *AMT1* gene transcription in the absence (0) or presence of 10 μM $CuSO_4$ for the indicated times. The *URA3* control and *AMT1* mRNAs are indicated with arrows. The fold induction of *AMT1* mRNA is shown at the bottom. (B) Cu resistance phenotypes of isogenic C. glabrata strains containing either the wild-type or *S16 AMT1* allele integrated at *ura3*. The isogenic wild-type (WT), *amt1–1*, *A16::URA3*, and *S16::URA3* strains were streaked onto SC agar and SC agar containing 20 or 100 μM $CuSO_4$. Plates were incubated at 30°C for 2 days and photographed. The genotypes of the four individual strains streaked onto each sector of the plates are indicated above. Terminal phenotype of C. glabrata cells harboring the S16 promoter mutation. C. glabrata cells harboring either the wild-type or S16 allele, integrated at *ura3*, were streaked to SC agar containing 100 μM $CuSO_4$. After a 24 hr incubation, the plates were photographed using a Zeiss Axioskop Photomicroscope at 200× magnification. A representative field for each strain is shown.

than that shown in Figure 1 because a suboptimal concentration of Cu was used for induction (see below).

Comparison of the ability of these two isogenic strains to grow in the presence of exogenous Cu demonstrated that the *A16::URA3* strain exhibited a Cu resistance profile indistinguishable from that of the wild-type parental strain, growing on media containing 100 μM $CuSO_4$ and in other experiments over 1.5 mM Cu (Figure 6B and data not shown). In contrast, although the *S16::URA3* strain was more resistant than the isogenic strain bearing a completely nonfunctional chromosomal *AMT1* gene (*amt1–1*), *S16::URA3* grew well on media containing up to 17.5 μM Cu but grew poorly on media containing 20 μM Cu and exhibited no colony formation at higher Cu concentrations (Figure 6B). Microscopic examination of cells streaked to agar containing 100 μM Cu, followed by a 24 hr incubation, demonstrated the presence of robust colonies for the *A16::URA3* strain that were indistinguishable from the wild-type strain. However, *S16::URA3* cells went through at most two cell divisions before reaching a terminal phenotype. Taken together, these results establish that rapid transcriptional activation of the *AMT1* gene via enhanced transcription factor access in the distorted nucleosome conferred by the homopolymeric (dA • dT) element is critical for normal cellular responses to the toxic metal Cu.

Discussion

A number of studies have demonstrated that chromatin structure has a potent positive or repressive impact on gene transcription via the modulation of protein–DNA or protein–protein interactions (Felsenfeld, 1992; Lewin, 1994; Paranjape et al., 1994; Kornberg and Lorch, 1995; Kingston et al., 1996; Struhl, 1996). Based on these investigations, chromatin structure within promoter regulatory regions has been classified as either preset or remodeled, two mechanisms that provide a means for access of transcription factors to *cis*-acting regulatory elements. Both of these forms of chromatin-mediated

regulation are established through protein-DNA interactions (Felsenfeld, 1992; Wallrath et al., 1994). In this work, we have demonstrated that a homopolymeric (dA • dT) element in the *AMT1* gene promoter plays a critical role in fostering rapid Cu-Amt1 protein binding to the metal responsive element and *AMT1* gene transcription in vivo. This represents a distinct mechanism for chromatin-mediated transcriptional regulation, which is conferred by intrinsic DNA structural features rather than through protein–DNA interactions. Although the data presented here provide strong evidence that the *AMT1* promoter region encompassing the A16 and MREs is packaged into a nucleosome in vivo, DNase I hypersensitivity flanking the A16 element in the wild-type promoter but not the scrambled *AMT1* promoter derivative strongly suggests that the homopolymeric (dA • dT) element locally disrupts the normal circular path of nucleosomal DNA (Richmond et al., 1984). This distortion of the DNA could weaken the interactions between the core histone proteins and the DNA region overlapping the metal response element within the *AMT1* promoter nucleosome. Consistent with a local rather than global deformation in the nucleosome structure, we observed that Cu-Amt1 binding to the MRE and gene activation do not lead to nucleosome disruption. This contrasts with the baker's yeast *PHO5* gene promoter, in which positioned nucleosomes are disrupted via the binding of Pho4p, thereby exposing the Pho2p-responsive *cis*-acting promoter element and the TATA box (Fascher et al., 1990). Furthermore, the homopolymeric (dA • dT) element-mediated distortion of the nucleosome in the *AMT1* promoter is distinct from the protein-mediated nucleosomal distortion, without disruption, observed for the mouse mammary tumor virus long terminal repeat. In this system, nuclear factor 1 (NF1) gains access to a *cis*-acting regulatory site embedded within a nucleosome via the prior binding of the glucocorticoid receptor (GR) to an exposed glucocorticoid response element (GRE) on the rotationally phased nucleosome. GR binding does not displace the nucleosome but is thought to locally perturb the structure of the nucleosome to facilitate the formation of the ternary complex between the nucleosome, GR, and NF1 (Truss et al., 1994). It is possible that, in cases where nucleosomal disassembly does not occur upon factor binding, the nucleosome plays an important positive role in gene expression or other genomic functions (Lewin, 1994; Wallrath et al., 1994). Since, in the linear DNA, the distance between the Amt1 binding site and the *AMT1* promoter TATA box is ~110 bp (Figure 3B), the presence of the nucleosome at the location determined here could serve to juxtapose DNA-bound Amt1 and the components of the transcriptional machinery bound at the TATA region. For example, nucleosome-mediated juxtaposition of the regulatory elements in the D. melanogaster *hsp26* promoter has been shown to play an important role in the heat shock transcriptional response (Lu et al., 1995).

Several observations in addition to those presented here are consistent with a model for a distorted *AMT1* promoter nucleosome and suggest a mechanism by which a homopolymeric (dA • dT) element might function in this manner. First, it has been established that the

Figure 7. Models for the Nucleosome within the Wild-Type and the Mutant *AMT1* Promoters

The blue cylinder represents the histone octamer and the maize tubing represents *AMT1* promoter DNA sequences. The homopolymeric (dA • dT) element, S16, and the Amt1-binding site are marked by A16, S16, and AMT1, respectively, and are positioned within the nucleosome to scale, according to the mapping data shown in Figure 3B. The two structural distortions of the DNA flanking the homopolymeric (dA • dT) element are proposed based on the DNase I hypersensitive sites flanking the A16 stretch shown in Figure 4. In the wild-type *AMT1* promoter containing the homopolymeric (dA • dT) element, the Amt1 binding site is highly accessible, whereas it is less accessible in the S16 mutant promoter.

reconstitution of DNA fragments containing homopolymeric (dA • dT) tracts into nucleosomes occurs with slightly less favorable $\Delta\Delta G$ (relative free energy differences) than that of DNA fragments containing a mixed sequence (Hayes et al., 1991; Puhl et al., 1991). Second, the crystal structure of six consecutive A-T base pairs reveals that the homopolymeric (dA • dT) element is straight, conformationally rigid, and contains additional non–Watson-Crick cross-strand hydrogen bonds (Nelson et al., 1987). Third, it has been demonstrated that homopolymeric (dA • dT) tracts are unbent in every crystal structure examined to date (Dickerson et al., 1996), consistent with a conformationally rigid structure. Together, these structural and biochemical features of homopolymeric (dA • dT) sequences could provide the basis for a DNA distortion within the nucleosome and overlapping the MRE. The rigidity of the homopolymeric (dA • dT) sequence may preclude this region of the DNA from conforming to the surface of the histone core, thereby resulting in reduced contacts between the *AMT1* promoter MRE and the histones in this region of the nucleosomal core particle. Although this has not yet been investigated, a localized region of reduced DNA-histone contacts could provide increased accessibility of Amt1 to the MRE. A working model for the A16-mediated localized nucleosomal distortion is shown in Figure 7.

A number of other examples of homopolymeric (dA • dT) elements residing in promoters occur in yeast and higher eukaryotic cells. Recently, a homopolymeric (dA • dT) element upstream of a Gcn4p binding site in the yeast *HIS3* gene was shown to stimulate Gcn4-dependent transcription and increase the access of Gcn4p to this DNA binding site in vivo (Iyer and Struhl, 1995). Iyer and Struhl proposed that the homopolymeric (dA • dT) sequence causes increased accessibility either through a local decrease in nucleosomal occupancy or an altered nucleosomal conformation. In this work, we have demonstrated that a homopolymeric (dA • dT) element is contained within a nucleosome and that this element provides the structural basis for rapid accessibility of a

metal-responsive transcription factor to its adjacent *cis*-acting regulatory element. Furthermore, the homopolymeric (dA • dT) element functions by conferring a local nucleosomal distortion without affecting overall nucleosome integrity. Iyver and Struhl (1995) suggested that such elements are likely to play important physiological roles in yeast cells and perhaps in the expression of genes in other organisms. Consistent with this notion, a large number of other yeast and higher eukaryotic gene promoters have been identified that contain homopolymeric (dA • dT) elements adjacent to known *cis*-acting regulatory elements. For example, the yeast *AAC3* gene, encoding a mitochondrial ADP/ATP translocator, contains a stretch of 17 contiguous A residues in the promoter region four nucleotides upstream of a binding site for Rox1, a protein involved in rapid transcriptional repression by oxygen (Zitomer and Lowry, 1992; Sabova et al., 1993). In mice, the Interleukin-5 gene promoter contains a stretch of 13 consecutive A residues 23 nucleotides upstream of a *cis*-acting regulatory site that binds NFIL-5P in response to phorbol ester or dibutyryl cAMP-stimulation of transcription (Lee et al., 1995). Therefore, the positioning of homopolymeric (dA • dT) elements, or other DNA elements with atypical structures, adjacent to *cis*-acting regulatory sites may play a key role in modulating the accessibility of transcription factors involved in gene activation or repression via the formation of specific nucleosomal structures.

In this work, we have shown that the homopolymeric (dA • dT) element, by virtue of creating a specific subnucleosomal structure that increases the access of Cu-Amt1 to the MRE, is critical for normal responses to toxic metal ions. C. glabrata cells harboring the S16 allele, as the sole source of the Amt1 protein, fail to mount a rapid transcriptional response and, as a consequence, are exquisitely sensitive to challenge with exogenous Cu. Since Cu readily engages in redox chemistry, which results in the generation of reactive oxygen species (Halliwell and Gutteridge, 1990), rapid transcriptional responses to Cu are important for protection from the toxic effects of oxygen-radical species and other forms of Cu-mediated cellular damage. This work directly links the rapid transcription of *AMT1* via a specialized nucleosome to the biology of Cu homeostasis in yeast cells. These observations suggest the possibility that other promoters utilize gene-specific nucleosome structures to regulate transcriptional responses to cellular stimuli.

Experimental Procedures

Strains and Growth Conditions

The isogenic C. glabrata strains 85/038, Q, and amt1–1 were previously described (Zhou and Thiele, 1993). C. glabrata strain 38236 is the wild-type parental strain for the generation of the spontaneous *ura3*$^-$ derivative, denoted D, by standard methods. For the construction of the C. glabrata strains A16::URA3 and S16::URA3, plasmids YIpAMT1::URA3 and pS16U1(b), respectively, were integrated in single copy at the *ura3* locus in strain amt1–1 as described (Zhou and Thiele, 1993). All strains were grown in synthetic complete medium (SC) or in SC lacking nutrients or containing $CuSO_4$, as specified in the figure legends.

Plasmids and RNA Analysis

Mutagenesis of the homopolymeric (dA • dT) element in the *AMT1* promoter was carried out using oligonucleotide-directed mutagenesis (Bio-Rad). The *AMT1* promoter was mutagenized in plasmid pBZ-12 and the mutations were verified by DNA sequence analysis and recombined with the unmanipulated *AMT1* promoter fragment, as a fusion to the E. coli *lacZ* gene, in plasmid YEp356R. The A16 element was deleted (A16Δ) and replaced with a sequence of 16 random nucleotides (S16) or 16 thymines (T16). The corresponding oligonucleotides were used in the mutagenesis experiments: 5′-ATTAGCTTATCATGACAGAATGTTAGTCTC-3′ (A16Δ), 5′-ATTAGCTTATCATGAAGCATGCGGATCCTGACAGAATGTTAGTCTC-3′ (S16), and 5′-ATTAGCTTATCATGAAAAAAAAAAAAAAAAAACAGAATGTTAGTCTC-3′ (T16). The corresponding lacZ fusion plasmids were denoted pBZ-45 (wild type; Zhou and Thiele, 1993), pRSA16Δ-lacZ, pRSS16-lacZ, and pRST16-lacZ. For in vivo footprinting and nucleosome-mapping studies, two plasmids, pRSAMT1 and pRSS16, were constructed by subcloning a 2.5 kb KpnI/XbaI DNA restriction fragment encompassing the wild-type *AMT1* gene or the S16 *AMT1* allele, respectively, into the KpnI/XbaI sites in the episomal plasmid pRS316. The plasmids YIpAMT1::URA3 and pS16U1(b) contain the wild-type or S16 alleles of the *AMT1* promoter and structural gene on the integrative plasmid pU1(b) (Zhou and Thiele, 1993). RNA expression from either the AMT1-lacZ fusion plasmids or the integrated *AMT1* alleles was assayed by RNase protection. C. glabrata cells were grown to log phase (OD_{650} 1.2 to 1.5), treated with $CuSO_4$ concentrations as indicated in the figure legends for varying lengths of time, harvested, and total RNA extracted as previously described (Koch and Thiele, 1996). Ten μg of RNA was used in each RNase protection reaction, and the C. glabrata *URA3* mRNA was measured as a control for the amount and integrity of RNA used in each RNase protection reaction. RNA levels were quantitated by phosphorImaging (Molecular Dynamics) and normalized to *URA3* levels.

In Vivo DMS Footprinting

The C. glabrata *ura3*$^-$ strain D, containing plasmids pRSAMT1 or pRSS16, were grown in SC-ura media to logarithmic phase at 30°C (OD_{650} 1.2 to 1.5), followed by $CuSO_4$ (100 μM) treatment for the time points indicated in the figure legends. Dimethyl sulfate (DMS) treatment was performed as described previously (Ganter et al., 1993). Cells were harvested and converted to spheroplasts and DNA was isolated using a Qiagen tip-100 as described (Qiagen). Isolated DNA samples were digested either with StyI or BstNI prior to G, A-specific DNA cleavage as described (Strauss et al., 1992), and the purified DNA was used in primer extension reactions as described below.

In Vivo Chromatin Analysis

C. glabrata cells were grown as described for in vivo DMS footprinting. After $CuSO_4$ (100 μM) induction at 30°C for 10 min, cells from 500 ml cultures were harvested and converted to spheroplasts in 30 ml of 1 M sorbitol, 1 mM EDTA, 10 mM DTT, and 4 mg/ml zymolyase (20T), with gentle shaking for 25 min at 30°C. The spheroplasts were resuspended in 12 ml of the appropriate buffer and treated with either DNase I (20–120 U/3 ml) or micrococcal nuclease (50–150 U/3 ml) as described previously (Ganter et al., 1993). DNA was purified and treated as described for DMS footprinting above.

In Vitro DNA Binding Studies

For in vitro DNA binding experiments, yeast extracts were prepared from C. glabrata D-strain cells untreated or treated with 100 μM $CuSO_4$ for 30 min as described (Company et al., 1988) and used in electrophoretic mobility shift assays (Koch and Thiele, 1996). A 110 bp fragment encompassing either wild-type (A16) or mutant promoter (S16) and intact *AMT1* MRE was ^{32}P-labeled by using polynucleotide kinase and [γ-^{32}P] ATP (Ausubel et al., 1987). DNA binding reactions were electrophoretically fractionated on a 6% native polyacrylamide gel.

In Vivo Restriction Endonuclease Accessibility Experiments

Cells were grown, harvested, and converted to spheroplasts as described above. The spheroplasts from 500 ml cultures were resuspended in 7.5 ml of 1 M sorbitol, 50 mM Tris–HCl (pH 8.0), and 10

mM $MgCl_2$ prewarmed at 30°C and mixed thoroughly with 7.5 ml of prewarmed AluI reaction buffer (New England Biolabs) of 1 M sorbitol, 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.4% NP-40, and 800 U of AluI (BRL), incubated at 30°C. At the time points indicated in the legend to Figure 5, 2 ml aliquots were transferred to 15 ml Falcon tube containing 1 ml of lysis buffer, mixed vigorously, and incubated at 65°C for 15 min. For Bsm AI digestion, a similar procedure was used except that the Bsm AI buffer (New England Biolabs) was used. The DNA was purified as described above and digested with either DraI or FokI restriction enzymes as controls.

Primer Extension Reactions

Five micrograms of DNA template, 4 pmol of ^{32}P 5′-end labeled *AMT1* promoter-specific primer, and 6 U of Vent (exo$^-$) DNA polymerase (New England Biolabs) were used in each reaction, with a total volume of 30 μl. The reaction buffer was previously described (Garrity and Wold, 1992). A linear amplification of 5 primer extension cycles was conducted in an automated thermal cycler (Perkin Elmer). The cycles were 5 min at 95°C, 10 min at the specific T_m for each oligonucleotide primer, and 3 min at 76°C for the first round and 2 min at 95°C, 10 min at T_m, and 3 min at 76°C for the following four rounds. The reactions were terminated, DNA purified as described (Ausubel et al., 1987) and electrophoretically fractionated on a 6% or 8% polyacrylamide-urea gel, and dried gels exposed to X-ray film or a phosphorImaging screen (Molecular Dynamics). The primers used in the extension reactions were: (P2) 5′-CCATAAAATG GACTAAACACTGGGC-3′, which hybridizes to *AMT1* promoter postions −136 to −160, (P4) 5′-CCTGCATTATTTGCGGGCAAGTT TCC-3′, −287 to −312, (P6) 5′-GGTGCTTTGTCTGGATTCTCAAC TAC-3′ from −51 to −76, (P10) 5′-CCGAGTGCCACTGAGAAAATG ACG-3′ from −240 to −263. Quantitation of all footprinting gels by phosphorImaging utilized control bands outside of the *AMT1* promoter as invariant standards for normalization.

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