The distal GATA sequences of the *sid1* promoter of *Ustilago maydis* mediate iron repression of siderophore production and interact directly with Urbs1, a GATA family transcription factor

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The *sid1* and *urbs1* genes encode L-ornithine N⁵-oxygenase and a GATA family transcription regulator, respectively, for siderophore biosynthesis in *Ustilago maydis*. The basic promoter and iron-regulatory sequences of the *U. maydis* *sid1* gene were defined by fusing restriction and *Bal31* nuclease-generated deletion fragments of the promoter region with the *Escherichia coli* β -glucuronidase (GUS) reporter gene. Sequences required for basal expression of *sid1* mapped within 1043 bp upstream of the translation start site and include the first untranslated exon and first intron. Sequences needed for iron-regulated expression of *sid1* were localized to a 306 bp region mapping 2.3 and 2.6 kb upstream of the ATG. The 306 bp region contains two G/TGATAA sequences, consensus DNA binding sites of GATA family transcription factors. Deletion or site-directed mutation of either or both GATA sequences resulted in deregulated expression of *sid1*. *In vitro* DNA binding studies showed that Urbs1 binds to the 3'-GATA site in the 306 bp iron-responsive region. However, deletion of 1.1 kb between the distal GATA sites and the basal promoter region led to deregulated expression of GUS, indicating that these GATA sequences are by themselves insufficient to regulate *sid1*. *In vitro* DNA binding and *in vivo* reporter gene analysis revealed that siderophores are not co-repressors of Urbs1.

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Introduction

Iron is one of the most abundant elements in nature, but its availability to living organisms is very limited due to the extremely low solubility of ferric ion ($[\text{Fe}^{3+}] = 10^{-18}$ M at pH 7) in aerobic environments (Griffiths, 1987; Williams, 1990). Ferrous ion under physiological conditions tends to oxidize, hydrolyze and polymerize, forming insoluble ferric hydroxide and oxyhydroxide polymers (Williams, 1990). On the other hand, too much cellular iron is harmful because iron possesses unfilled

d atomic orbitals and is able to undergo changes in oxidation state involving one electron. This easy access to two oxidation states allows iron to react with oxygen and produce hydroxyl radicals (Halliwell and Gutteridge, 1984; Imlay and Linn, 1988). Hydroxyl radicals react at an extremely high rate with most organic molecules found in cells, in particular, attacking and destroying cell membranes and DNA (Halliwell and Gutteridge, 1984).

To acquire iron from the environment and, at the same time, avoid toxicity from excess cellular iron, organisms have developed various elaborate biological systems to coordinate iron transport and homeostasis (Griffiths, 1987). In plants, iron is transported in the sap in the form of complexes with low molecular weight chelators, such as citrate and malate. In mammals, iron is mobilized to different tissues via the iron binding protein transferrin. In egg white, iron is stored in the iron binding protein ovotransferrin. Most microorganisms produce and secrete a class of low molecular weight ferric iron chelating agents termed siderophores to gather environmental iron (Bagg and Neilands, 1987; Mei and Leong, 1994). In general, siderophore biosynthetic genes are negatively regulated by iron (Bagg and Neilands, 1987; Mei and Leong, 1994). One of the few microorganisms which do not produce siderophores is the yeast *Saccharomyces cerevisiae* (Klausner and Dancis, 1994). In *S. cerevisiae*, several factors, including two membrane-bound ferric reductases FRE1 (Dancis *et al.*, 1992) and FRE2 (Georgatsou and Alexandraki, 1994), a ferric ion transporter, FTR1 (Stearman *et al.*, 1996) and a membrane-associated multicopper oxidase/ferroxidase FET3 (Askwith *et al.*, 1994; DeSilva *et al.*, 1995), work together to mediate iron uptake under iron starvation conditions. Like siderophore biosynthetic genes, FRE1, FRE2, FTR1 and FET3 are negatively regulated by iron (Dancis *et al.*, 1992; Askwith *et al.*, 1994; DeSilva *et al.*, 1995). This regulation is mediated by AFT-1 (Yamaguchi-Iwai *et al.*, 1996), a transcription activator that binds to the promoters of these genes in the absence of iron.

Ustilago maydis produces two cyclic hexapeptide siderophores, ferrichrome and ferrichrome A (Budde and Leong, 1989). The first step in ferrichrome and ferrichrome A biosynthesis is catalyzed by ornithine N⁵-oxygenase (Wang *et al.*, 1989; Mei *et al.*, 1993). The gene encoding ornithine N⁵-oxygenase (*sid1*) has been characterized (Mei *et al.*, 1993). Siderophore biosynthesis in *U. maydis* is negatively regulated by the iron concentration in the growth medium. Urbs1, a transcription regulator of siderophore biosynthesis in *U. maydis*, contains two finger motifs characteristic of the GATA family of transcription factors (Voisard *et al.*, 1993). Disruption of *urbs1* leads to constitutive expression of *sid1* (Mei *et al.*, 1993), suggesting that Urbs1 may directly interact with the *sid1* promoter to repress biosynthesis of siderophores in *U. maydis* when cellular iron is not limiting.

In this communication we describe the structural analysis of the *sid1* promoter and document the specific interaction of Urbs1 with sequences in this promoter. Two GATA motifs 1.6 kb upstream of the transcription initiation site in the *sid1* promoter are required for iron-regulated expression of *sid1*. *In vitro* electrophoretic gel mobility shift assay indicated that Urbs1 interacts directly with one of the GATA sequences. Finally, a model involving DNA looping for the regulatory action of Urbs1 on *sid1* expression is discussed.

Results

sid1 promoter analysis

To delineate the functional domains in the *sid1* promoter region, seven convenient restriction fragments in the *sid1* promoter region (pCG2.8, pCGC, pCG2.0, pCG1.6, pCGPN, pCGS1 and pCGPS) were fused with the GUS reporter gene (Gallagher, 1992), which encodes the *E. coli* β -glucuronidase in the *U. maydis* self-replicating vector pCM54 (Tsukuda *et al.*, 1988; Figure 1A). Both wild-type UM001 (strain 518) and a *urbs1* disruption mutant UMC015 were transformed with these constructs and β -glucuronidase activities of the transformants were determined on plates. The results of these experiments are summarized in Figure 1A. A 0.7 kb fragment between 2.3 (pCG2.0) and 3 (pCG2.8) kb upstream of the translation start site was required for iron-regulated expression of *sid1*. The basic promoter activity was mapped within 1.27 kb (pCGS1) upstream of the translation start site. Sequences containing the 654 bp intron which is located between the transcription and translation start sites of *sid1* were also required for basal expression of the gene (pCGPN and pCGPS). Deletion of the -2285 to -1267 region (pCGC), which is located downstream of the 700 bp iron-responsive sequence and upstream of the basic promoter of *sid1*, abolished iron-regulated expression of *sid1*. Fusions between GUS and promoter fragments in the wrong orientation were constructed as controls. None of these constructs gave GUS activity (Figure 1A).

To further define the promoter region of *sid1*, a set of progressive deletions of the 3 kb *sid1* promoter region was created by *Bal31* nuclease treatment (p1007–p1044; Figure 1B). The sequence for basal expression of the gene was further mapped within a 228 bp fragment between -1271 (pCGS1) and -1043 bp (p1028) upstream of the translation start site (Figure 1A and B). Sequences required for iron-regulated expression of *sid1* were localized to a 306 bp region mapping 2.3 (pCG2.0) and 2.6 (p1011) kb upstream of the ATG (Figure 1A and B). This 306 bp fragment contains two G/TGATAA sequences separated by 23 bp (Figure 1C).

To eliminate copy number and position effects, three *Bal31* nuclease-generated promoter–GUS constructs (p1010, p1013 and p1028; Figure 1B) were introduced into the *sid1* locus of *U. maydis* by gene replacement. GUS activity of strain UMS113, which has the reporter gene under the control of the 2.7 kb *sid1* promoter region, was repressed 16-fold in cells grown on high iron medium (Figure 1D). When a fragment containing the two upstream G/TGATAA sequences was removed from the *sid1* promoter in strain UMS115, GUS activity became constitutive and was no longer regulated by iron concentration in the

medium (Figure 1D). In strain UMS117, the 228 bp fragment required for basal expression of *sid1* was deleted. As expected, no GUS activity was detected in this strain (Figure 1D). Results from these experiments confirmed the observations made with fusion constructs in the self-replicating vector and GUS plate assay.

Epitope tagging of *urbs1* and detection of Urbs1 protein in *U. maydis*

To confirm that *urbs1* encodes an expressed protein in *U. maydis*, the influenza hemagglutinin antigen (YPYDVP-DYA)₃ was translationally fused as an epitope at the C-terminus of Urbs1. The epitope-tagged Urbs1 was constructed in the *U. maydis* self-replicating vector pCM54 to give pAN15. Biological activity of the epitope-tagged Urbs1 was tested by its ability to complement the *urbs1* gene disruption mutant UMC015. On high iron medium, UMC015 gives an orange colony phenotype due to the constitutive production of siderophore by this strain. In comparison, UMC015/pAN15 transformants, which carry the epitope-tagged Urbs1, displayed a complemented wild-type white colony phenotype on high iron medium (data not shown). This indicates that the epitope-tagged Urbs1 is biologically active in *U. maydis*. pAN15 replicates as a multicopy plasmid; therefore, epitope-tagged Urbs1 is expressed in multiple copies in UMC015/pAN15 transformants. To avoid copy number effects, U^{Urbs1}^{tag}, in which the wild-type *urbs1* allele was replaced by the epitope-tagged allele, was constructed. Siderophore production by U^{Urbs1}^{tag} in both low and high iron medium was first determined qualitatively using the color plate assay. Wild-type strain 518, siderophore constitutive production mutant UMC015 and an *urbs1* NTG mutants UMC002 were included as controls. The results showed that siderophore production in 518 and U^{Urbs1}^{tag} is negatively regulated by iron, as indicated by their white colony phenotype on high iron medium (data not shown). In contrast, the two *urbs1* mutants, UMC015 and UMC002, displayed an orange colony phenotype on high iron medium (data not shown). Siderophore production by these strains was also determined quantitatively in both low and high iron media (Table I). These studies further indicate that epitope-tagged Urbs1 was biologically functional in *U. maydis*. A protein of ~110 kDa, the predicted molecular weight of epitope-tagged Urbs1, was detected in *U. maydis* expressing epitope-tagged Urbs1 (UMC015/pAN15) by immunoprecipitation/Western blotting using a monoclonal antibody (anti-Ha) to influenza hemagglutinin antigen and protein A–Sepharose (Figure 2). No signal was detected in cells expressing wild-type Urbs1 (UMC015/pSC3; Figure 2). Urbs1^{tag} was detected at similar levels in both low-iron and high-iron growth conditions (Q. Zhao and S.A. Leong, unpublished findings).

Evidence for direct interaction of Urbs1 with the *sid1* promoter by electrophoretic gel mobility shift analysis

Since the 306 bp DNA fragment containing the iron-responsive sequence(s) in the *sid1* promoter region has two GATA sites and *urbs1* encodes a GATA family transcription factor with two putative zinc finger domains, Urbs1 may act as a transcription repressor of *sid1* by binding to GATA motifs in the *sid1* promoter. *In vitro*

Fig. 1. *sid1* promoter analysis. (A) Restriction fragments of the 2.9 kb *SspI*–*PvuII* *sid1* promoter were fused to the GUS reporter gene carried on the *U.maydis* replicating vector pCM54 and transformed into *U.maydis* wild-type (528) and an *urbs1* disruption mutant (UMC015). GUS activity was determined colorimetrically as described in Materials and methods, on E medium containing no added iron or 10 μ M FeSO_4 . + indicates that strong activity (colonies turned dark blue) and – indicates that no activity (colonies remained white) was seen within 6 h after application of X-gluc to colonies. This assay is only a qualitative measure of promoter activity. (B) A set of progressive deletions of the 3 kb *sid1* promoter region created by *Bal31* nuclease treatment were fused to the GUS reporter gene. Transformation and GUS activity assay were the same as in (A). (C) Sequence of the iron-responsive region. (D) GUS activities of gene replacement strains carrying the wild-type and deleted *sid1* promoters fused to GUS. β -Glucuronidase activities were determined fluorometrically as described in Materials and methods, in whole cell extracts obtained from UMS113, UMS115 and UMS117 cells cultured on minimal liquid medium containing no added iron or 10 μ M FeSO_4 . Assays were done in triplicate on five independent gene replacement strains for each construct. Activities were corrected by subtracting the fluorescence observed using an extract of the untransformed strain 518, which gave similar levels of background fluorescence at 0 and 30 min to that observed at time 0 for extracts of the gene replacement strains. The filled boxes for p1011 (A) and UMS113 (D) indicate the location of GATA sequences required for iron-regulated expression of GUS, and the GATA boxes are underlined in the sequence showed in (C).

Table I. Siderophore production^a by wild-type and *urbs1* mutants

	Low iron	High iron	Low iron/high iron ratio
518	0.46 ± 0.02	0.03 ± 0.00	15
UMC015	0.88 ± 0.04	0.26 ± 0.02	3
UMC002	0.71 ± 0.09	0.26 ± 0.01	3
UMurbs1 ^{tag}	0.25 ± 0.01	0.04 ± 0.01	7

^aCells were grown for 48 h at 28°C in low iron medium with or without 10 µM FeSO₄. Culture supernatants were treated with an equal volume of ferric perchlorate solution and the absorbance was read at 495 nm (Budde and Leong, 1989). Analyses were done in triplicate. Data were normalized to equal cell growth using an adsorbance at OD₆₀₀ of 1.0.

Fig. 2. Detection of Urbs1 in *U. maydis*. Immunoprecipitation of epitope-tagged Urbs1 and detection of Urbs1 in *U. maydis* by Western blotting as described in Materials and methods. UMC015 is an *urbs1* disruption mutant. pSC3 carries the wild-type *urbs1* gene while pAN15 carries the tagged *urbs1* gene on the *U. maydis* replicating vector pCM54.

DNA binding studies were conducted with the 306 bp GATA-containing DNA fragment and whole cell extracts of UMC015, a *urbs1* disruption mutant, and UMC015, carrying a replicating plasmid expressing the wild-type Urbs1 (pSC3) or epitope-tagged Urbs1 (pAN15). To induce Urbs1 to be in an active conformation for siderophore gene repression, whole cell extracts were isolated from cultures grown in high iron medium (10 µM FeSO₄). As shown in Figure 3, the 306 bp fragment was shifted to give one major band by extracts obtained from strains expressing *urbs1* and epitope-tagged *urbs1*, but not by extracts from the *urbs1* null mutant. To determine whether the shifted bands were due to Urbs1, a monoclonal antibody (anti-Ha) to the influenza hemagglutinin epitope was included in the gel shift reactions. As shown in Figure 4, the addition of increasing amounts of antibody reduced the level of the shifted bands in the assays containing the tagged Urbs1 but not in assays containing wild-type Urbs1, indicating that Urbs1 is associated with the gel shift complex. No supershifted band was observed under the conditions of electrophoresis employed.

To determine whether the GATA boxes in the 306 bp fragment are the *in vitro* DNA binding sites for Urbs1, a

Fig. 3. DNA binding assays of the 0.3 kb DNA fragment which contains the two distal GATA sites in the iron-responsive sequence of the *sid1* promoter region with whole cell protein extracts of UMC015, UMC015/pSC3 and UMC015/pAN15. UMC015 is an *urbs1* disruption mutant. pAN15 carries the *urbs1* tagged gene on the *U. maydis* replicating vector pCM54 and pSC3 carries wild-type *urbs1* on the same vector. Electrophoretic gel mobility shift assays were done as described in Materials and methods.

Fig. 4. DNA binding assays of UMC015/pSC3 and UMC015/pAN15 whole cell protein extracts and the 0.3 kb *sid1* promoter probe with increasing amounts of monoclonal antibody (anti-Ha) to the influenza hemagglutinin antigen as indicated. Strains and conditions were as described in Figure 3 and in Materials and methods.

63 bp oligonucleotide (oligonucleotide 1 in Figure 5A) encompassing the two distal GATA sequences was synthesized and used for DNA-protein binding assays with whole cell extracts of UMC015 and UMC015/pSC3. The results showed that the 63 bp oligonucleotide was shifted by UMC015/pSC3 extract, which expresses wild-type *urbs1*, but not by extract from the *urbs1* null mutant UMC015 (Figure 5B). The specificity of DNA binding

Fig. 5. Electrophoretic gel mobility shift analysis of Urbs1 and target DNA. (A) Oligonucleotides used in gel shift analysis. Sequences of the 63 bp oligonucleotides containing the wild-type or mutated distal GATA sites. Numbers indicate the distance of the G residues from the translation start of *sid1*: 1, wild-type; 2, 5'-GATA mutant; 3, 3'-GATA mutant; 4, double GATA mutant. (B) Specificity of DNA binding of the wild-type 63mer [oligonucleotide 1 in (A)] with whole cell protein extract of UMC015/pSC3. Different amounts of unlabeled oligomer (0, 10, 20 and 30 ng) were added as indicated. Strains and conditions as described in Figure 3 and in Materials and methods. (C) Gel shift assays of UMC015/pSC3 whole cell protein extracts with the 63 bp oligonucleotide and its mutant variants shown in (A). Lane 1, wild-type; lane 2, 5'-GATA mutant; lane 3, 3'-GATA mutant; lane 4, double GATA mutant. Strains and conditions were as described in Figure 3 and in Materials and methods.

was examined by including unlabeled oligonucleotides in the DNA–protein binding reactions. The binding signal between the labeled probe and Urbs1 was reduced by the addition of excess unlabeled probe in the reaction (Figure 5B).

The role of the two GATA motifs in the 63 bp oligonucleotide probe was assessed by mutation of one or both sites in the probe (oligonucleotides 2, 3 and 4 in Figure 5A). Mutation of the 5'-GATA site had no apparent effect on the *in vitro* binding of Urbs1 to the probe, while mutation of the 3'-GATA site or mutation of both GATA sites eliminated binding (Figure 5C). To test the *in vivo* roles of the two GATA sites, three *sid1* promoter–GUS constructs which carry mutations at the 5'-GATA (pAY2), 3'-GATA (pAY3) or both (pAY4) sites were introduced into the *sid1* locus of *U.maydis* by gene replacement to give UMS202 (5'-GATA to CTGA), UMS203 (3'-GATA to CTGA) and UMS204 (double mutation). GUS activities expressed by these three strains were determined in both low and high iron growth conditions. UMS113, which has

Table II. β -Glucuronidase activity produced from wild-type and mutant *sid1* promoters fused to the GUS gene^a

	Low iron	High iron	Low iron/high iron ratio
UMS113	15.7 $\pm$ 1.8	1.0 $\pm$ 0.04	16
UMS202	13.7 $\pm$ 3.3	4.9 $\pm$ 0.9	3
UMS203	17.9 $\pm$ 1.7	10.4 $\pm$ 1.6	2
UMS204	19.8 $\pm$ 3.0	18.8 $\pm$ 1.7	1

^aWild-type *sid1* promoter and *sid1* promoters containing distal GATA mutations fused with GUS were introduced into the *sid1* locus by gene replacement. The genotype of each strain is described in Table III. Assays were done in triplicate on three independent transformants and the experiment was repeated three times. Assay conditions for GUS activity are described in Figure 1 and Materials and methods.

the reporter gene under the control of the wild-type *sid1* promoter region, was included as a control. The results are shown in Table II. When the 5'-, 3'- or both GATA sites were mutated to CTGA in the *sid1* promoter region in strains UMS202, UMS203 and UMS204, GUS activity was no longer tightly regulated by iron concentration in the medium, as indicated by the low –Fe: +Fe ratio (Table II). Mutation of a single GATA site led to partial deregulation of GUS activity, while mutation of both sites led to complete deregulation of GUS activity. In contrast, the GUS activity in strain UMS113, which has the reporter under the control of the wild-type *sid1* promoter, was negatively regulated by iron concentration in the medium (Table II).

Discussion

This study represents the first comprehensive analysis of a *U.maydis* gene promoter. The complexity of the *sid1* promoter provides a unique opportunity to understand better how *U.maydis* promoters and transcription factors function. By using GUS as a reporter, three regions in the 3 kb promoter have been defined to be important for expression of *sid1*. The first region is a 306 bp fragment encompassing two GATA sequences and mapping 1.6 kb from the start of transcription. Deletion of the GATA sequences resulted in deregulated expression of *sid1* by iron. This result indicated that these GATA sequences might be iron-responsive elements and the DNA binding sites for Urbs1. Since these two GATA motifs are distantly located from the transcription start site, we refer to them as the 'distal GATA sites'. A second region of 228 bp located 211 bp upstream of the start of transcription is required for basal level expression of the gene. A CAAT box and pyrimidine-rich sequence are both located in this region (Mei et al., 1993). A third region encompassing the first exon and intron of *sid1* is also required for basal level expression of the gene. This finding is not surprising, since introns contribute to basic promoter activity as well as transcription regulation in other genes (Crestani et al., 1993; Brown and Taylor, 1994; Corrochano et al., 1995). However, a definitive role for the intron in expression of *sid1* will require further mutational studies.

In vitro electrophoretic gel mobility shift analysis using synthetic oligonucleotides containing the wild-type and mutated distal GATA sites indicate that Urbs1 binds specifically to the distal 3'-GATA sequence located 1.6 kb

Fig. 6. A proposed transcription 'looping' model for the regulatory action of Urbs1 on *sid1* expression. (A) In the presence of iron, Urbs1 interacts with the two distal GATA sites and one or more of the downstream GATA sites (the two proximal GATA sites were used in the Figure) to form a loop in the *sid1* promoter region. Deletion of the distal sites (B) or alteration of the spacing of the binding sites by deletion of the -2285 to -1267 region (C) prevents loop formation, leading to constitutive expression of *sid1*. The two open boxes located -2472 and -2443 upstream of the ATG are the two distal GATA sites (D). The two open boxes located -875 and -850 upstream of the ATG are the two proximal GATA sites (D). The filled boxes indicate a putative transcription factor binding site (D).

upstream of the transcription start site of the *sid1* promoter region. Site-directed mutagenesis indicated that not only the distal 3'-GATA site but also the distal 5'-GATA site in the iron-responsive region is required for iron-mediated transcription repression of *sid1* *in vivo*. This is not surprising, as high affinity binding sites in promoters are not always correlated with the biological function of transcription factors (A. Laughon, personal communication). However, the possibility that mutation of the 5'-GATA site disrupts binding of a second factor required for iron regulation of *sid1* cannot be discounted. DNase protection studies will shed further light on the extent of the region bound by Urbs1.

The distal arrangement of the iron-responsive elements in *sid1* suggests that Urbs1 may effect a novel mechanism of transcription repression in *sid1*. In addition to the two distal GATA sequences, there are additional GATA sequences in the *sid1* promoter region. These include the two proximal GATA sites (Figure 6) and GATA sites in the first intron (5'-CTATCG-182 bp-CGATATCT-3'). Some of these GATA sites may also play a role in Urbs1-mediated regulation of *sid1*. Even though the two distal GATA sites are required for iron-regulated expression of *sid1*, the presence of the distal GATA sites is not sufficient to mediate repression of *sid1*, as deletion of the 1018 bp *HindIII*-*SacI* fragment in the promoter region resulted in deregulated expression of *sid1* (Figures 1A and 6C). This suggests that spacing of the distal GATA sites relative to some other site(s) in the promoter is critical and/or that deletion of the *HindIII*-*SacI* fragment eliminated another sequence(s) that is essential for iron-mediated regulation of *sid1*. It is interesting to note that this region contains the palindromic GATA sequence TTTTATCAGAT, which matches 11 bp of the distal 5'-GATA site perfectly and might serve as a binding site for Urbs1. However, this site is isolated from other full GATA sites. The GATA

family transcription factor Nit2 shows a clear preference for binding sites containing two closely apposed GATA elements (Xiao and Marzluf, 1996). Moreover, GATA-1 interactions with palindromic GATA sites are considerably stronger than with those having single GATA elements (Trainor *et al.*, 1996); yet strong binding of Urbs1 to the 5' distal GATA site was not observed. As a result, we believe that this GATA site may not play an essential role in regulation of *sid1*. Mutation of this site and analysis of its ability to interact specifically with Urbs1 are underway to confirm this interpretation.

Based on the results available, we propose a model predicting that Urbs1 mediates the formation of a DNA loop in the 1.6 kb promoter region of *sid1* which involves the distal GATA and the proximal GATA sites or the two GATA sites in the intron (Figure 6A and D). DNA looping has been reported in the regulation of a large number of prokaryotic and eukaryotic genes (Bellomy and Record, 1990; Schleif, 1992). One of the best studied examples is the AraC-regulated *BAD* operon in *E. coli* (Dunn *et al.*, 1984; Martin *et al.*, 1986; Huo *et al.*, 1988). In eukaryotic systems, DNA looping has been shown to play a role in silencing of the mating type genes in the yeast *Saccharomyces cerevisiae* (Hofmann *et al.*, 1989) and enhancer-mediated control of the prolactin gene in the rat (Cullen *et al.*, 1993). Sequences required for basal expression of *sid1* are located between the upstream and downstream GATA sites (Figure 6). Formation of a loop between the upstream and downstream GATA sites could impede the activity of basic transcription factors either through steric hindrance or direct interaction of transcription factors with Urbs1 (Herschbach *et al.*, 1994) or by forming a topologically isolated sequence that can no longer bind transcription factors. Mutational studies of the proximal and intronic GATA sites are in progress to assess their role in regulation of *sid1*. However, loop formation might not involve the interaction of Urbs1 bound to both downstream and distal GATA sites, but rather the interaction of Urbs1 bound to distal GATA sites with another factor bound to the downstream promoter region. In this regard, it is interesting to note that GATA-1 can interact physically with the transcription factors Sp1 and EKLF and that Sp1 is able *in vivo* to recruit GATA-1 to a promoter in the absence of GATA binding sites (Merika and Orkin, 1995). A comprehensive analysis of the DNA looping model for the regulatory function of Urbs1 is underway to investigate these possibilities.

Since the function of Urbs1 as a transcription repressor of *sid1* is mediated by cellular iron concentration, iron may play a direct or indirect role in the conformation, function and/or cellular localization of Urbs1. Efforts are underway to investigate the functional association of iron with domains of Urbs1 purified from *U. maydis* and to study the subcellular location of Urbs1 and the *in vivo* binding of Urbs1 to DNA in iron-starved and iron-replete cells. Results from two experiments suggest that siderophores do not act as co-repressors of Urbs1. *In vitro* DNA binding analysis showed that neither deferrated nor ferrated ferrichrome (present at 1 µg/µl) affected *in vitro* binding of Urbs1 and the distal GATA site of the *sid1* promoter (data not shown). Moreover, expression of the GUS gene in UMS113 was negatively regulated by the iron concentration of the medium (Figure 1D), indicating

Table III. *Ustilago maydis* and *E.coli* strains used in this study

Strain name	Source	Genotype
<i>Ustilago maydis</i>		
518	Holliday, 1974	Wild-type <i>a2 b2</i>
UMC002	Voisard <i>et al.</i> , 1993	<i>urbs1 a2 b2</i>
UMC015	this study	<i>a2 b2 urbs1::phleo^r</i>
UMSH ^r 008	Mei <i>et al.</i> , 1993	<i>a2 b2 sid1::hyg^r</i>
UMS113	this study	<i>a2 b2 sid1::GUS phleo^r</i>
UMS115	this study	<i>a2 b2 sid1::GUS phleo^r</i>
UMS117	this study	<i>a2 b2 sid1::GUS phleo^r</i>
UMS202	this study ^a	<i>a2 b2 sid1-202::GUS phleo^r</i>
UMS203	this study ^a	<i>a2 b2 sid1-203::GUS phleo^r</i>
UMS204	this study ^a	<i>a2 b2 sid1-204::GUS phleo^r</i>
UMurbs1 ^{tag}	this study	<i>a2 b2 urbs1::tag hyg^r</i>
<i>Escherichia coli</i>		
DH5 α	BRL Inc., Gaithersburg, MD	f80dlacZ DM15 <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA96 r^- F^-</i> $\Delta(lacZYA-argF)U169$

^aGene replacement strains carrying site-directed mutations of the distal GATA sequences in the *sid1* promoter. Mutations 202, 203 and 204 are shown in Figure 5.

that siderophores are not involved in iron-mediated regulation of the *sid1* promoter by *Urbs1*. Since the *sid1* structural gene is replaced by the GUS reporter gene, no siderophores are produced in strain UMS113.

Materials and methods

Ustilago maydis and *E.coli* strains

The strains used in this paper are listed in Table III. UMC015, a gene disruption mutant of *urbs1*, was constructed as follows: The internal 2 kb *StuI* fragment in pSC3 (Voisard *et al.*, 1993) was replaced by blunt end ligation with the 2 kb *XbaI*–*HindIII* fragment of pUble20 containing the Tn5-*phleo* *U.maydis* expression cassette (Gold *et al.*, 1994) to give pUWAN41. The *XbaI*–*BamHI* fragment of *urbs1* in the genome of strain 518 (UM001) was replaced by double cross-over with the *XbaI*–*BamHI* fragment of pUWAN41 containing the Tn5-*phleo* cassette. Gene replacements were scored by screening for the orange phenotype of *urbs1* mutants on high iron medium (Voisard *et al.*, 1993) and confirmed by Southern hybridization analysis.

Three *Bal31* nuclease-generated *sid1* promoter–GUS fusion constructs from p1010, p1013 and p1028 were introduced into the *sid1* locus by double cross-over to give strains UMS113, UMS115 and UMS117 respectively. These constructs were done as follows. The *XbaI*–*SspI* fragment which contains upstream DNA flanking the *sid1* locus from pUWAN2 was cloned at the *SacII* site of pBSKS(+) by blunt end ligation to give pUWAN110. The *PvuII*–*EcoRI* fragment which contains the downstream DNA flanking the *sid1* locus from pUC(7.5) was cloned into the *XbaI* site of pUWAN110 by blunt end ligation to give pUWAN111. The *XbaI* fragments which contain various *sid1* promoter sequences fused to GUS from p1010, p1013 and p1028 were cloned into the *NotI* site of pUWAN111 by blunt end ligation to give pUWAN113, pUWAN115 and pUWAN117, respectively. The *KpnI*–*XbaI* fragments of pUWAN113, pUWAN115 and pUWAN117 were co-transformed with the *HindIII* fragment of pUble20, which contains the Tn5-*phleo* *U.maydis* expression cassette, into UMSH^r008, which carries a *hyg^r* marker at the *sid1* locus. Gene replacement events were scored by screening for *phleo^r*, *hyg^r* and confirmed by Southern hybridization analysis.

UMS202 UMS203 and UMS204, which contain the GATA mutated *sid1* promoter–GUS fusion constructs in the *sid1* locus were constructed by the same strategy as used for the construction of UMS113, UMS115 and UMS117. Briefly, The *SspI*–*PvuII* fragments containing site-directed mutations in the GATA sites were fused to GUS at the *BamHI* site in pCG. The *EcoRI*–*XbaI* fragments were cloned into the *NotI* site of pUWAN111 by blunt end ligation to give pAY2 (5'-GATA mutation), pAY3 (3'-GATA mutation) and pAY4 (double mutation). The *KpnI*–*XbaI* fragments of pAY2, pAY3 and pAY4 were used for transformation.

In UMurbs1^{tag}, the *urbs1* locus was replaced by epitope-tagged *urbs1*. This strain was constructed by co-transforming UMC015 with the *XbaI*–*SmaI* fragment in pUWAN13 and *BamHI*-linearized pHL1 (Wang *et al.*, 1988), which contains the *U.maydis* *hyg^r* expression cassette. Gene

replacement events were scored by screening for *phleo^r*, *hyg^r*, white colony phenotype on high iron medium (Voisard *et al.*, 1993) and confirmed by Southern hybridization analysis.

Subclones of the *sid1* locus

The 2.2 and 7.5 kb *HindIII* fragments of pSidI (Wang *et al.*, 1989; Mei *et al.*, 1993) were cloned into the *HindIII* site of pUC18 to give pUCHIII2.2 and pCU(7.5) respectively. The 6.4 kb *BglII* fragment was cloned into the *BamHI* site of pCM54 (Tsukuda *et al.*, 1988) to give pCMBII. The 8.7 kb *EcoRI* fragment of pSid1 was cloned into the *EcoRI* site of pUWAN1 to yield pUWAN2. pUWAN1 was created by removing the pUC18 116 bp *SspI*–*AatII* fragment, Klenow filling and religation.

Construction of GUS reporter gene fusions with various restriction fragments of the *sid1* promoter region

The 1.8 kb *XbaI*–*BamHI* fragment of pNOM123 (a gift of R.P.Oliver) containing the promoterless GUS structural sequence was cloned into the *XbaI* and *BamHI* sites of pCM54 backbone (Tsukuda *et al.*, 1988) to give pCG. DNA restriction fragments isolated from pUCHIII2.2 or pCMBII were blunt ended and cloned into the unique *BamHI* site of pCG by blunt end ligation to give pCG1.6 (1.6 kb *NcoI*–*PvuII*), pCGPN (0.9 kb *NcoI*–*PvuII*), pCGPS (0.5 kb *SacI*–*PvuII*), pCG2.0 (2 kb *HindIII*–*PvuII*), pCG2.8 (2.8 kb *SspI*–*PvuII*) or pCGS1 (1.2 kb *SacI*–*PvuII*). pCGC was constructed by deleting the 1 kb internal *HindIII*–*SacI* fragment of pCG2.8. pCG2.8 (–), pCGC (–), pCG2.0 (–) and pCG1.6 (–) were controls having the respective fragments cloned in the opposite orientation.

Plasmids p1007–p1044 were a set of progressive deletions of the 2.9 kb *SspI*–*PvuII* *sid1* promoter region fused to GUS created by *Bal31* nuclease treatment. p104, the plasmid used for the *Bal31* digestion, was constructed as follows: The *SspI*–*PvuII* fragment from pUWAN2 was cloned into the *EcoRV* site of pBSKS(+) (Stratagene, La Jolla, CA) to give pUWAN101 the *PvuII* fragment of pUWAN101 was inserted into the *SspI* site of pUWAN2 to give pUWAN102. The *PvuII*–*SacII* fragment of pUWAN102 was inserted at the *BamHI* site of pCG by blunt end ligation to give pUWAN104. The *KpnI*-linearized pUWAN104 DNA was then digested with *Bal31* nuclease. The *Bal31* nuclease reaction products were digested with *SpeI* and blunt end ligated. Desired plasmids were selected by restriction analysis and DNA sequencing.

Construction of epitope-tagged *urbs1*

To construct the epitope-tagged *urbs1* allele, a *NotI* site was introduced at the C-terminus of *urbs1* by PCR using two synthetic oligonucleotides: P42 (5'-CCAGATCTCAGCGGCCCGCCCTGGCAGCGAAGCC-ATCCGC-3'), corresponding to the C-terminal sequence of *urbs1* and also containing a *NotI* site, a stop codon and a *BglII* site; P43 (5'-GTCGTCCCATGGCACCAG-3'), corresponding to a region upstream of the C-terminus of *urbs1* and spanning a *NcoI* site. PCR products from oligonucleotides P42 and P43 using pSC6 (Voisard *et al.*, 1993) as template were digested with *NcoI* and *BglII* and ligated to the large

NcoI–*Bgl*III fragment of pSC6 to give pSC6Not. The 114 bp *NorI* fragment from pGTEP1 containing a triple influenza hemagglutinin epitope was then inserted in-frame at the *NorI* site of pSC6Not to give pUWAN13. The epitope-tagged *urbs1* was subsequently cloned into the *U. maydis* self-replicating vector pCM54 by replacing the *XbaI*–*Bgl*III fragment of pSC3 (Voisard *et al.*, 1993) with the *XbaI*–*Bgl*III fragment of pUWAN13 to give pAN15. Orientations of inserts in all the constructs were determined by restriction enzyme digestion and/or DNA sequencing.

Site directed mutagenesis

The Muta-Gene phagemid *in vitro* mutagenesis kit from BioRad (Hercules, CA) was employed. The 4.4 kb *Bam*HI fragment of pUWAN2, which contains the *sid1* promoter region, was cloned into the *Bam*HI site of pTZ18U to give pUWAN81. The mutagenic primers used to introduce mutations in the GATA sites were oligonucleotides 2, 3 and 4 in Figure 5A for mutation in 5'-GATA, 3'-GATA and both GATA sites, respectively. Mutations were confirmed by DNA sequencing.

Reagents and transformation techniques

A microfuge tube-based procedure (Wang *et al.*, 1988; Voisard *et al.*, 1993) was employed for *U. maydis* transformation. Standard *E. coli* procedures were used for all bacterial work (Ausubel *et al.*, 1994). Restriction endonucleases and modifying enzymes were from NEB (Beverly, MA). All reagents from other sources are identified in the text.

Complementation of *urbs1* mutants by the C-terminus-tagged *urbs1*

Wild-type strain 518, *urbs1* disruption mutant (UMC015) and *urbs1* NTG mutant (528) were transformed with C-terminus-tagged *urbs1* (pAN15) and the non-tagged *urbs1* to *hyg^r*. Siderophore production was determined quantitatively in liquid medium by ferric perchlorate assay and qualitatively on solid medium by colony color assay as described previously (Budde and Leong, 1989; Mei *et al.*, 1993; Voisard *et al.*, 1993).

Preparation of *U. maydis* cellular extracts and SDS–PAGE of protein

Ustilago maydis cells grown at 28°C in 250 ml low iron medium with 10 µM FeSO₄ for 3 days were harvested and washed with HEPES buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10 mM phenylmethylsulfonyl fluoride). Washed cells were resuspended in 1.5 ml HEPES buffer and disrupted by passage through a French pressure cell at 12 000 p.s.i. and then centrifuged at 15 000 g for 20 min at 4°C. Total protein in the supernatant was determined by the Bradford method (Ausubel *et al.*, 1994). A total of 20 µg cellular protein was loaded for each sample and run on 8×10×0.07 cm 0.7% SDS–polyacrylamide gels at 10 mA for 2 h. Gels were stained with Coomassie brilliant blue.

Immunoprecipitation of epitope-tagged *Urbs1* and Western blotting

NaCl was added to 1 mg total cellular protein in 500 µl HEPES buffer to a final concentration of 500 mM. Five micrograms of anti-Ha (12CA5) monoclonal antibody (Boehringer Mannheim, Indianapolis), dissolved in TBS buffer (20 mM Tris, pH 7.5, and 500 mM NaCl), was then added to the mixture. The mixture was rotated overnight at 4°C followed by the addition of TBS-equilibrated protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C. The beads were washed five times with TBS buffer. After washing with TBS buffer, the beads were resuspended in 20 µl 2× SDS gel loading buffer and boiled for 5 min. The supernatant was subjected to SDS–PAGE.

Proteins in the gel were transferred onto nitrocellulose filters by electrophoresis at 4°C in Tris–glycine buffer at 50 V for 6 h. Protein standards transferred onto nitrocellulose filters were stained with Ponceau S and marked. Remaining protein binding sites on the nitrocellulose filters were blocked with 5% non-fat dried milk in 1× TBS buffer at room temperature for 4 h, followed by immunoblotting with 5 µg anti-Ha (12CA5) monoclonal antibody in 1 ml 5% non-fat dried milk at room temperature for 8 h. The blots were washed with 1× TBS and incubated with 5 µl anti-mouse IgG alkaline phosphatase conjugate (Sigma, St Louis, MO) in 5 ml 5% non-fat dried milk at room temperature for 2 h. After the final wash with 1× TBS buffer, the filters were transferred to BCIP/NBT solution (Sigma) for enzymatic detection of conjugated antibody.

DNA binding assays

The 0.3 kb *XbaI*–*Hind*III DNA fragments from p1011 used in the DNA binding assay were end-labeled with [α -³²P]dCTP and Klenow fragment.

The sequences of the oligomers used in the DNA binding assay are listed in Figure 5A.

Oligomers were first end-labeled with [γ -³²P]dATP and T4 DNA kinase and then annealed with cold complementary oligomers to generate double-stranded DNA probes. For each DNA binding reaction, labeled DNA (1–2 ng, 2–3×10⁴ c.p.m.) was incubated in 20 µl DNA binding buffer containing 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 2 mM MgCl₂, 0.3 mg/ml bovine serum albumin, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 µg poly(dI–dC) (Peleg and Metzberg, 1994) and 5 µg *U. maydis* protein extract. Electrophoresis was carried out at 10 V/cm at 4°C using a 1:1 dilution of TBE (100 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) as running buffer. Gels were pre-electrophoresed for 2 h before samples were loaded. Following electrophoresis, gels were transferred to Whatman 3 MM paper, dried under vacuum at 80°C and subjected to autoradiography.

β -Glucuronidase assay

Plate assays for β -glucuronidase activity in *U. maydis* (Gallagher, 1992; Naleway, 1992) were done colorimetrically on E medium (Wang *et al.*, 1989) with or without the addition of 10 µM FeSO₄. Cells were grown overnight in 1 ml liquid E medium and then 10 µl spotted on solid E medium. When the colony size reached ~3 mm in diameter, 2 µl X-gluc (50 mg/ml in dimethylformamide) were spotted on top of the colony and the colonies were incubated at 28°C for color development. Quantitative assay of β -glucuronidase activity in whole cell extracts prepared from liquid cultures was done fluorometrically using 4-methylumbelliferyl β -D-glucuronide (MUG) as substrate (Gallagher, 1992). Cells from 5 ml culture (low iron with or without 10 µM FeSO₄) were collected by centrifugation at 3340 g for 5 min and resuspended in GUS extraction buffer (50 mM NaHPO₄, pH 7.0, 10 mM β -mercaptoethanol, 10 mM Na₂EDTA, pH 8.0, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). The cells were quickly frozen in liquid nitrogen and then slowly thawed on ice. An equal volume of glass beads was added to the thawed cells and the cells were broken by vortexing (30 s vortexing/min on ice, repeated five times). The supernatant was collected after centrifugation at 23 300 g for 5 min. All steps were carried out on ice or at 4°C.

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