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DNA-binding properties of the yeast Rgt1 repressor

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Abstract

The yeast *HXT* (glucose transporter gene) repressor Rgt1 lacks a dimerization domain and thus appears to bind as a monomer to its consensus binding site sequence ($_{5'}$CGGANNA$_{3'}$). The *HXT1* promoter contains 8 Rgt1 binding sites, but its expression is not effectively repressed by Rgt1. In the present study, the Rgt1 binding sites in the *HXT1* promoter were analyzed to examine how Rgt1 mediates transcriptional repression. I show that Rgt1 binds the *HXT1* promoter, but does not significantly mediate repression. When engineered to be multimerized without the intervening sequences between the Rgt1 binding sites, however, 4 or more Rgt1 binding sites were required to provide sufficient Rgt1-dependent repression. These findings suggest that the intervening sequences between the Rgt1 binding sites are important for the regulation of Rgt1 function and that Rgt1 functions efficiently only through multiple binding sites.

Introduction

In the budding yeast *S. cerevisiae*, expression of the glucose transporter gene (*HXT*) is regulated by glucose; *HXT* expression is induced by high levels of glucose, and repressed when glucose levels are low [1,2]. Repression of *HXT* expression appears to be mediated by the DNA-binding protein Rgt1, which binds and recruits the general corepressors Ssn6 and Tup1 to the *HXT* promoters [3–5]. Rgt1 binds DNA through its N-terminal domain containing a Zn_2Cys_6 binuclear cluster, one of the most common DNA-binding domains in the fungal DNA binding proteins [4,5]. Many of the proteins containing this cluster, such as Gal4, Ppr1, and Put3, appear to bind as dimers to 2 ‘CGG’ sequences, separated by variable length spacers. Crystal structures of the proteins revealed that a leucine zipper-like coiled-coil motif mediates the homo-dimerization of each of the proteins, enabling them to specifically recognize their binding sites [6–7]. The *Aspergillus nidulans* transcriptional activator AlcR belongs to the zinc binuclear cluster protein family, but lacks the dimerization domain. Thus, AlcR binds DNA as a monomer to its single binding sites. AlcR, however, activates transcription only if multiple sites are present [8].

Like AlcR, Rgt1 lacks the coiled-coil dimerization domain and binds DNA motifs that contain a ‘CGG’ sequence, but not those containing a pair of these sequences [3,4,9]. These observations suggest that Rgt1 binds DNA as a monomer. Footprinting analysis of the upstream regions of *HXT* genes indicated that Rgt1 specifically recognizes the ‘CGGANNA’ sequence (N is any nucleoside) [5]. Rgt1 appears to bind cooperatively to multiple copies of the sequence, which provides a way for Rgt1 to limit its binding to the

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yeast genome. Different *HXT* promoters contain different numbers of Rgt1 binding sites; within 1000 bp upstream of the ATG start codon, the *HXT1* promoter contains 8 sites, whereas the *HXT2* and *HXT4* promoters contain 3 sites. Moreover, in the absence of glucose, repression of the expression of the *HXT2* and *HXT4* genes is completely relieved by the deletion of *RGT1*, whereas *HXT1* expression is still significantly repressed in an *rgt1* mutant [10]. Therefore, Rgt1 functions differently at different promoter regions. Here, the DNA-binding properties of Rgt1 were examined to gain more insight into how Rgt1 functions through its binding sites.

Materials and Methods

DMS footprinting assay

The *HXT1* upstream region from −400 to −550 was PCR-amplified and labeled with ^{32}P as described previously [5]. For dimethylsulfate (DMS) protection footprinting [11], Rgt1 was incubated with ^{32}P-labeled DNA fragments (2×10^5 cpm) in 20 μl of TGZD buffer (20 mM Tris-HCl, pH. 8.0, 75 mM KCl, 10 μM $ZnCl_2$, 5% glycerol) containing 1μg of poly(dI-dC)-poly(dI-dC) at room temperature for 20 min. Rgt1-DNA complexes were incubated with 0.2% DMS (final concentration) at 20°C for 1 min. The methylated bases were cleaved by boiling DNA in PE buffer (10% piperidine and 10 mM EDTA) at 90°C for 30 min.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as described previously [5]. Briefly, yeast cells expressing Rgt1-HA [12] were treated with formaldehyde (1%, final concentration) and cell extracts were prepared by vortexing cell pellets with glass beads in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate). After centrifugation, the cell lysates were sonicated five times with 10-s pulses using a microtip. Fragmented chromatins were precipitated with monoclonoal HA antibody. The cross-linking of the precipitated DNA to the protein was reversed by incubating them in elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA) at 65°C for 6 h.

Functional analysis of Rgt1 binding sites

Gene expression reporter plasmids containing Rgt1-binding sites were prepared by inserting various Rgt1 binding sites between the *LEU2* upstream activation sequence and the TATA box of *HIS3* fused to *lacZ* (pBM2832) [4]. Clusters of the Rgt1 binding sites in the *HXT1* promoter were PCR-amplified. Multiple copies of Rgt1 binding sites without intervening sequences between the sites were prepared by annealing complementary oligonucleotides containing different numbers of Rgt1 binding site (CGAATCCGGAA AAACTACG).

Results and Discussion

Characterization of Rgt1 binding to the HXT1 promoter

DMS is a small molecule that penetrates the protein-DNA complex and predominantly methylates the N-7 atoms of guanines in the major groove of B-DNA [11]. To probe the interactions between Rgt1 and the CGG triplet in the Rgt1 binding sites ($_{5'}$CGGANNA$_{3'}$), Rgt1-DNA complexes were treated with DMS and the resulting methylated bases were cleaved with piperidine. Footprinting analysis indicated that Rgt1 protects the central guanine residues corresponding to positions −451G and −481G (Fig. 1A), and that, like most other zinc cluster proteins, Rgt1 directly interacts with the CGG triplet. The *HXT1* promoter contains 8 Rgt1 binding sites within 1000 bp upstream of the ATG start codon [5]. To detect Rgt1 binding to its sites *in vivo*, a chromatin immunoprecipitation assay of the *rgt1* mutant expressing *RGT1-HA* [12] was performed using a monoclonal HA antibody. Rgt1 was shown to be associated with the 3 clusters of Rgt1 binding sites in the *HXT1*

promoter (I, II, and III; Fig.1B). There are 3 Rgt1 binding sites in the *HXT2* (at −577, −430, and −393) and *HXT4* (at −645, −424, and −295) promoters (numbers are the positions of the central G residues of the CGG triplet in relation to the ATG start codon). The individual Rgt1 binding sites in the *HXT2* and *HXT4* promoters could be amplified by polymerase chain reaction from chromatin immunoprecipitates, suggesting that Rgt1 can bind to its single binding sites (data not shown).

Functional analysis of the Rgt1 binding sites in the HXT1 promoter

To determine whether the Rgt1 binding sites in the *HXT1* promoter are equally functional, the 3 clusters of Rgt1 binding sites were amplified by polymerase chain reaction and inserted into the promoter of the *HIS3-lacZ* reporter, and the constructs were expressed in wild-type and *rgt1* mutant strains (Fig. 1C). Clusters I and II contained 2 Rgt1 binding sites that were 36 bp apart, but only binding to cluster II provided ~13-fold repression by Rgt1. This finding suggests that the intervening sequences between Rgt1 binding sites are involved in regulating *HXT1* expression. When 4 Rgt1 binding sites were present, the reporter genes induced 33- (P5) and 54-fold (P4) repression by Rgt1, suggesting that Rgt1 binding on multiple sites synergistically mediates repression. Importantly, the full-length *HXT1* promoter (P6) containing 8 Rgt1 binding sites induced about 100-fold repression, but Rgt1 appeared to mediate only 10% of the repression. These findings suggest that Rgt1 binds to the *HXT1* promoter, but does not significantly mediate repression, and indicate that the intervening sequences between the Rgt1 binding sites have an important role in lowering the efficiency of Rgt1-dependent repression.

Rgt1 functions efficiently through its multiple binding sites

The questions of whether elimination of the intervening sequences between the Rgt1 binding sites increases the efficiency of Rgt1-dependent repression and the number of Rgt1 sites required to support sufficient Rgt1-dependent repression were examined next. To this end, complementary oligonucleotides containing different numbers of Rgt1 binding sites were inserted into the *HIS3-lacZ* promoter and the resulting constructs were expressed in yeast cells under the repressing condition. The activity of the synthetic DNA sequences was first tested by an electrophoretic mobility shift assay (EMSA) using the amino-terminal fragment of Rgt1 that contains a zinc binuclear cluster domain [5] (Fig. 2A). The results of the gel shift assay (EMSA) indicated that the truncated Rgt1 could bind to the DNA fragment containing an Rgt1-binding site, but bound with stronger affinity to the DNA probes that contained multiple binding sites. Further, the rate of migration of the DNA-Rgt1 complexes through polyacrylamide gel was progressively retarded with an increase in Rgt1 concentration, suggesting that the truncated Rgt1 fragment does not cooperatively bind DNA *in vitro* as reported previously [5]. A functional test of the synthetic, multimerized Rgt1 binding sites revealed that reporter genes containing 2 or 3 Rgt1 binding sites provided 2- to 3-fold repression, whereas those containing 4 and 6 sites induced about 30- and 90-fold repression, respectively (Fig. 2B). Rgt1 cooperatively binds to the cluster of 5 Rgt1 binding sites in the region between −290 to −400 in the *HXT3* promoter *in vivo*, leading to an approximately 57-fold repression [5]. Therefore, the synergistic repression by RGt1 binding to multiple Rgt1 binding sites without intervening sequences is probably due to efficient recruitment of Rgt1 to the sites. These findings suggest that Rgt1 binds to single binding sites, but functions more efficiently through its binding to multiple sites, and that the DNA-binding properties of Rgt1 are similar to those of AlcR [8].

Yeast *HXT* promoters regulated by Rgt1 have different numbers of Rgt1 binding sites, and different effects on Rgt1-dependent repression. For example, the *HXT2* and *HXT4* promoters contain 3 Rgt1 binding sites spaced 100~200 bp apart (Fig. 2C), and repression is completely relieved in an *rgt1* mutant [4]. Rgt1-dependent repression appears to be more

effective in the *HXT2* and *HXT4* promoters with fewer Rgt1 binding sites than in the *HXT1* promoter with more Rgt1 binding sites. Therefore, Rgt1 functions differently at different promoters, perhaps due to different architecture of the Rgt1 binding sites in the promoters.

Conclusion

The separation of multimers of potential binding sites by intervening sequences is a common feature of regulatory proteins in natural promoters [13]. The *HXT1* promoter contains 8 binding sites for the *HXT* repressor Rgt1, which does not significantly mediate the repression of *HXT1* expression. The intervening sequences between the Rgt1 binding sites in the *HXT1* promoter, however, likely play a regulatory role in Rgt1-dependent repression. A yet unidentified regulatory protein(s) may bind to the intervening sequences to influence the Rgt1-dependent repression. This view is supported by the finding that Rgt1 is efficiently recruited to multiple copies of the Rgt1 binding sites without intervening sequences and mediates synergistic repression of transcription.

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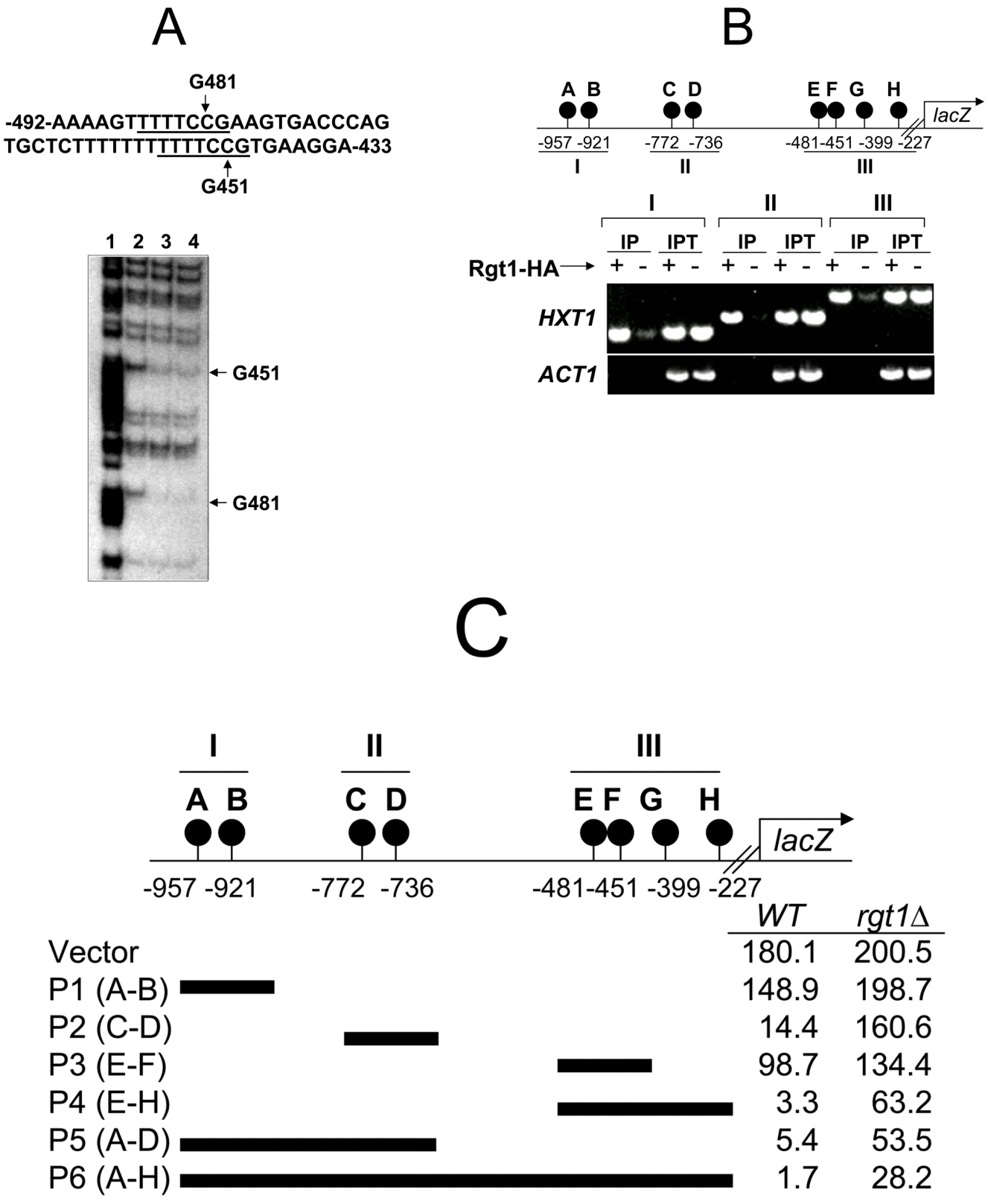


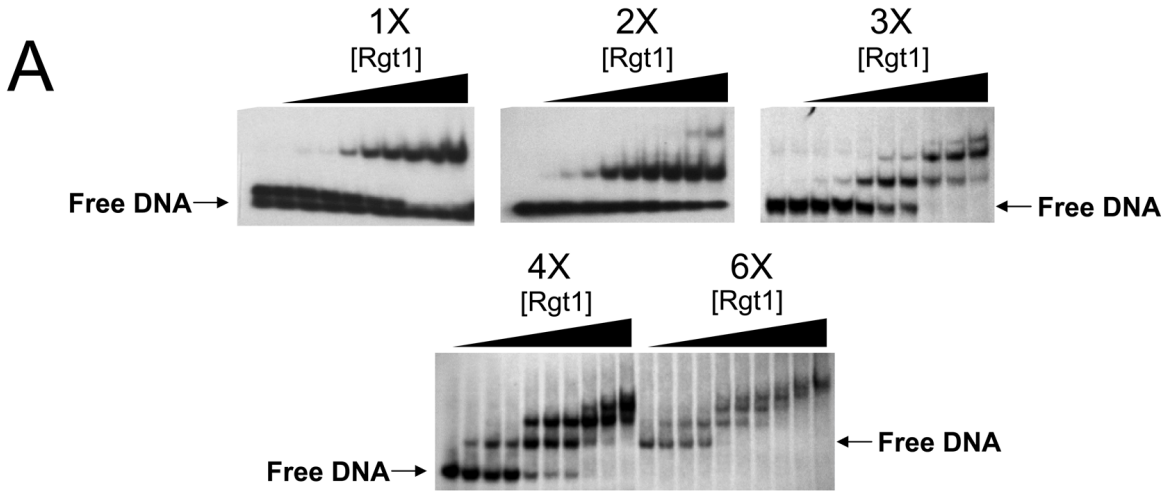
Fig. 1.

Rgt1 binds to the *HXT1* promoter, but does not sufficiently mediate transcriptional repression.

A. Binding of Rgt1 to the *HXT1* promoter *in vitro*. Top: The sequence of the *HXT1* upstream region between −433 and −492. The Rgt1 binding sites (CGGANNA) were underlined. Bottom: *In vitro* methylation protection assay of the *HXT1* upstream regulatory region (−400 to −550) containing two Rgt1 binding sites. The first 188 amino acids of Rgt1, encompassing the Zn_2Cys_6 DNA-binding domain in its N-terminus, fused to the glutathione-*S*-transferase was expressed in *Escherichia coli* BL21 (DE3) and was affinity-purified using the glutathione agarose beads [5]. Gel lanes: 1; A+G sequencing ladder, 2; without Rgt1, 3; 100 ng Rgt1, 4; 300 ng Rgt1.

B. Binding of Rgt1 to the *HXT1* promoter *in vivo*. The *rgt1* mutant expressing HA-Rgt1 (+) or an empty plasmid (−) was grown to mid-log phase (OD_{600}=1.0~1.2) in YP medium containing 2% galactose and subject to ChIP assay using the HA antibody. Rgt1 binding sites were PCR-amplified using different primer sets covering different regions of the promoter. IP; immunoprecipitated, IPT; input.

C. The upstream region of *HXT1* was divided into three sections (cluster I, II and III) and inserted between the *LEU2* upstream activation sequence and the TATA box of *HIS3* fused to *lacZ* in pBM2832 [4]. β-Galactosidase activity was assayed using the yeast β-galactosidase assay kit (Pierce) and activities were presented in Miller units [5]. Yeast cells were grown to mid-log phase (OD_{600}=1.0~1.2) in synthetic medium containing 2% galactose.



B

β-galactosidase activity

No. of the Rgt1 site	*WT*	*Rgt1Δ*
0	180.1	200.5
1X	143.4	170.5
2X	86.2	160.3
3X	58.3	170.6
4X	6.2	156.2
6X	2.1	200.8

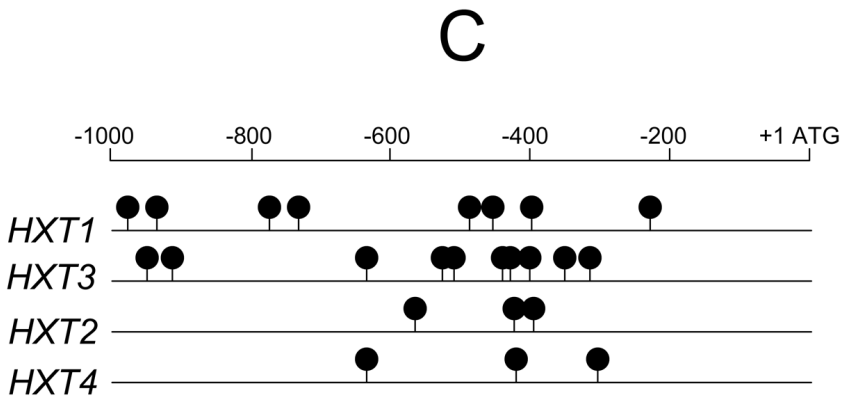


Fig. 2.

Rgt1 is efficiently recruited by artificially multimerized binding sites without intervening sequences and mediate synergistic repression of transcription.

A. Electromobility shift assay with the ^{32}P-labelled oligonucleotides containing different numbers of Rgt1 binding sites (1x, 2x, 3x, 4x, and 6x) and the different amounts of N-terminal fragment of Rgt1 (0, 2.5, 5, 10, 20, 40, 80, 120, 250, and 500 ng per lane, from the left).

B. The complementary oligonucleotides containing different numbers of the Rgt1 binding sites were inserted between the *LEU2* upstream activation sequence and the TATA box of *HIS3* fused to *lacZ* [4]. β-Galactosidase activity was assayed as described in Fig. 1.

C. Different *HXT* promoters have different numbers of the Rgt1 binding sites. Black circles indicate positions of the Rgt1 consensus binding site sequence ($_{5'}$CGGANNA$_{3'}$).