A C-terminal Segment with Properties of α-Helix Is Essential for DNA Binding and in Vivo Function of Zinc Finger Protein Rme1p*

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Rme1p plays important roles in the control of meiosis and in cell cycle progression through binding to upstream regions of IME1 and CLN2 in Saccharomyces cerevisiae. Rme1p has three zinc finger segments, and two of them are atypical. To determine DNA binding domain of Rme1p, a series of Rme1p derivatives fused with maltose-binding protein were purified and characterized by gel mobility shift assay. We show that not only three zinc fingers, but also the neighboring C-terminal region is essential for DNA binding. Mutational analysis of this region revealed that basic residues Arg-287, Lys-290, and Arg-291 and the hydrophobic residues Phe-288, Leu-292, Ile-295, and Leu-296 are critical for DNA binding. In addition, double substitutions by proline at Asn-289 and Lys-293, each of which was not essential for DNA binding, abolished DNA binding. These results suggest that the C-terminal segment forms an amphipathic helical structure. Furthermore, it was shown that the mutations in the important basic residues abolish or impair Rme1p function in vivo for repression and inhibition of spore formation. Thus, the C-terminal segment is essential and acts as a novel accessory domain for DNA binding by zinc fingers.

Zinc finger proteins are a major class of eukaryotic transcription factors (1–4), and recent analysis of the human genome sequence revealed that 564 genes, ~1.5–2.0% of the genes in human, encode C_{2}H_{2} zinc finger proteins (5). Many zinc finger proteins recognize specific DNA sequences (1–4), although this domain also participates in protein-RNA and protein-protein interactions (6–8). Structural features and functional requirements for the binding of C_{2}H_{2} zinc fingers to DNA have proven valuable for understanding biochemical and biological regulatory mechanisms. In addition, this information has had significant impact in protein engineering, permitting the design of novel DNA-binding proteins useful for diagnostics and gene therapy (3, 4). However, the interaction of zinc finger domains with DNA is not simple, and general rules for DNA recognition are continually refined and extended. Thus, there are still significant challenges in understanding zinc finger protein-DNA interaction and in developing design methods for zinc finger proteins with novel specificity.

Our studies focus on the zinc finger protein Rme1p, which has a vital role in the life cycle of Saccharomyces cerevisiae (9–12). It prevents haploid cells from initiating meiosis, a specialized division that would be nonproductive and potentially lethal. Rme1p has two characterized molecular roles. First, it represses IME1, which specifies an activator of a major class of meiosis-specific genes (13–16). Second, it stimulates expression of CLN2 (17), which specifies a mitotic G_{1} cyclin. A very similar G_{1} cyclin (ClnP) has been shown to exclude Ime1p from the nucleus (18). Therefore, Rme1p may inhibit meiosis through two convergent mechanisms.

We have characterized the action of Rme1p at the IME1 promoter, which is unusual among yeast promoters for its size, complexity, and repression mechanism (14–16, 19, 20). Rme1p binds to two sites upstream of IME1 at ~2030 and ~1950 in vivo and in vitro, and both sites are required for repression of IME1 (14, 16, 19). As expected for a zinc finger DNA-binding protein (29), treatment of the purified Rme1p with EDTA abolishes its DNA binding activity (16). The nature of Rme1p-DNA recognition is of interest for two reasons. First, Rme1p has three zinc fingers (designated ZF1, ZF2, ZF3; see Fig. 1) and yet protects over 13 bp as determined by in vitro and in vivo footprinting (14, 16). Second, two of the Rme1p zinc fingers are atypical (13) as seen in Fig. 1B. ZF1 has a C_{2}H_{2}E_{1} structure rather than a C_{2}H_{2} structure. ZF2 lacks the first hydrophobic residue, and the number of residues between the second cysteine and the second hydrophobic residue exceed the consensus (X_{6} instead of X_{5}; see Fig. 1B). Also, the ZF1–ZF3 linker region contains 21 amino acids rather than the 5 residues found in the most common linker arrangement. To determine the molecular consequences of these novel features, we have initiated a study of the Rme1p structural requirements for DNA binding. We find that the C-terminal region adjacent to ZF3 is required for DNA binding. Mutational analysis supports a model in which this region forms an amphipathic α-helix, which may contact DNA directly.

**EXPERIMENTAL PROCEDURES**

Construction and Purification of Rme1p and Its Mutant Proteins Fused with MBP—Full-length and truncated RME1 genes (RME1(171–300), RME1(171–284), RME1(171–237), RME1(201–300), and RME1(201–284)), the numbers in parentheses show the position numbers of the amino acid residues in Rme1p; also see Fig. 1) were amplified by polymerase chain reaction from plasmid pAM240 (13) as a template, which carries RME1 on an ~3-kilobase pair Sau3A1-BgIII fragment in the pBR322 BamHI site. The polymerase chain reaction fragments

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1 The abbreviations used are: ZF, zinc finger; bp, base pair(s); MBP, maltose-binding protein; RC, repression cassette; C-TR, C-terminal region.

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FIG. 1. A, schematic representation of Rme1p and its deletion mutant proteins fused with MBP. DNA binding ability was determined by gel mobility shift assay using a 404-bp IME1 upstream DNA fragment (−1746 to −2146) as shown in Fig. 2. B, amino acid sequence alignment of three zinc fingers of Rme1p (178−300 residues) with the consensus sequence of C2H2 zinc finger. X indicates any amino acid, and ψ is a hydrophobic residue. Amino acids residues matched with the CH2 consensus sequence are underlined. Based on the three-dimensional structures of zinc fingers determined (1, 2), putative β-stand and α-helix regions in the Rme1p zinc fingers are assigned as indicated by the gray arrows and the white cylinder, respectively. The numbers −1, 2, 3, and 6 correspond to the positions relative to the start of the α-helix. The C-TR region is also indicated.

were inserted between the Xmr1 and HindIII sites of pMAL-c2 (New England Biolabs) to fuse with the malE gene that encodes maltose-binding protein (MBP). Site-directed mutagenesis in the C-terminal region was introduced by two-step polymerase chain reaction from the plasmid carrying the RME1-171-300 gene in pMAL-c2. The sequences for RME1 and its mutants in all the constructs were verified by DNA sequencing.

Plasmids constructed were introduced in the Escherichia coli TG1 strain, and MBP-Rme1p proteins were overexpressed in 1 liter of LB medium containing 100 μg/ml ampicillin and 0.01 mM ZnCl2 by the addition of 0.5 mM isopropyl-1-thio-b-D-galactopyranoside at A600 = 0.5. The cell lysate was loaded onto an amylose-resin chromatography column, MBP-Rme1p was eluted by the column buffer containing 10 mM maltose according to the manufacturer’s protocol. The purity of MBP-Rme1p was checked by 10% SDS-polyacrylamide gel electrophoresis at 50 V. The concentration of purified MBP-Rme1p was determined by the Bradford method (21) using bovine serum albumin as a standard.

Gel Mobility Shift Assay—The 404-bp fragment of IME1 upstream (−2140 to −1746) was subcloned into the EcoRI site of a pBR322 derivative plasmid (16). The 404-bp RC fragment was isolated from low melting temperature agarose gel electrophoresis of the EcoRI digests of the plasmid, and it was radioactively labeled with treatment with Klenow fragment together with [α-32P]dATP. 0.16 pmol of 32P-labeled fragment was mixed with a different amount of proteins (0.16 pmol) in 30 μl of the reaction buffer (50 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.5 mM MgCl2, 1.5 mM dithiothreitol, 8% glycerol) containing 2 μg poly(dI-dC)poly(dI-dC) and 7.5 μg of bovine serum albumin. After incubation for 30 min on ice, each mixture was loaded and run on a 6% native polyacrylamide gel electrophoresis at 5°C (16). The DNA bands were visualized by autoradiography.

Miscellaneous Methods—S. cerevisiae strains have genetic markers ura3-52 leu2 trpl lys2 ho::LYS2 and are isogenic except as noted. Strains AMP119 (a rme1Δ::LEU2), AMP112 (a rme1::Pgal::S53-RME1-TRP1 gal80::LEU2), and AMP1124 (a rme1::Pgal::S53-RME1-213-TRP1 gal80::LEU2) were described previously (14–16). Strains MHS11 (a rme1::Pgal::S53-RME1-171-300::TRP1 gal80::LEU2), MHS13 (a rme1::Pgal::S53-rme1::K290A/R291S/K293A/K294A-pMAL-c2-rme1::TRP1 gal80::LEU2), and MHS15 (a rme1::Pgal::S53-rme1::K290A-pMAL-c2-rme1::TRP1 gal80::LEU2) were described previously (14–16). Strains MHS11 (a rme1::Pgal::S53-RME1-171-300::TRP1 gal80::LEU2), MHS13 (a rme1::Pgal::S53-rme1::K290A/R291S/K293A/K294A-pMAL-c2-rme1::TRP1 gal80::LEU2), and MHS15 (a rme1::Pgal::S53-rme1::K290A-pMAL-c2-rme1::TRP1 gal80::LEU2) were described previously (14–16). Strains MHS11 (a rme1::Pgal::S53-RME1-171-300::TRP1 gal80::LEU2), MHS13 (a rme1::Pgal::S53-rme1::K290A/R291S/K293A/K294A-pMAL-c2-rme1::TRP1 gal80::LEU2), and

β-Galactosidase and sporulation assay results are the average of three independent cultures of each strain.

RESULTS

Characterization of DNA binding of Truncated Rme1p Mutant Proteins by Gel Mobility Shift Assay—Rme1p consists of 300 amino acid residues, and three regions similar to C2H2 zinc fingers lie at positions 178−199 (ZF1), 206−234 (ZF2), and 256−281 (ZF3) (13) as shown in Fig. 1. For analysis of DNA binding by Rme1p, we purified a series of Rme1p derivatives (Fig. 1A) as fusions to E. coli MBP. DNA binding affinity was characterized through gel mobility shift assays using a 404-bp IME1 upstream fragment (−2146 to −1743) that contains two Rme1p binding sites. With full-length MBP-Rme1p (Fig. 2A), we observed one complex at low [protein]/[DNA] ratios (lanes 5–7). A second complex forms at higher [protein]/[DNA] ratios (lanes 8–10), which we have shown to depend upon presence of two Rme1p binding sites in the template (16).

To determine whether the N-terminal region of Rme1p is required for DNA binding, we examined the N-terminal deletion derivative MBP-Rme1p71−300 (Fig. 1A). As seen in Fig. 2B, this fusion protein binds the DNA fragment with affinity similar to MBP-Rme1p, forming a second complex at nearly the
same [protein]/[DNA] ratio as that of full-length MBP-Rme1p. Competition assays indicate that MBP-Rme1p<sub>171–300</sub> binds to each site in the IMEI upstream region with similar affinities (data not shown) as determined previously for MBP-Rme1p<sub>16</sub>. Thus, the truncated MBP-Rme1p<sub>171–300</sub> retains the full binding specificity of intact Rme1p. Further truncation to yield MBP-Rme1p<sub>201–300</sub>, which lacks ZF1, abolished DNA binding. Similarly, MBP-Rme1p<sub>284–300</sub>, which lacks ZF3, did not bind to DNA (summarized in Fig. 1A). This result is in keeping with the finding that point mutations at cysteine residues in any of the Rme1p zinc fingers abolish DNA binding (13, 14). We conclude that DNA binding by Rme1p does not require the N-terminal region (residues 1–170) and does require the zinc finger region. Consistent with a property of zinc finger DNA-binding proteins (29), incubation of the protein with 10 mM EDTA inhibits the DNA binding activity of Rme1p<sub>171–300</sub> (data not shown) as does the full-length Rme1p<sub>16</sub>.

To determine whether the three zinc fingers are sufficient for DNA binding, MBP-Rme1p<sub>171–284</sub> was constructed and characterized. This protein lacks both the Rme1p N-terminal region, and a short C-terminal region. MBP-Rme1p<sub>171–284</sub> did not form detectable protein-DNA complexes (Fig. 2C), indicating that the C-terminal region (residues 285–300; called the C-TR) is essential for DNA binding. These results indicate that the zinc fingers are not sufficient for DNA binding ability. Thus, the DNA binding domain of Rme1p consists of three zinc fingers and the C-TR.

**Mutational Analysis of C-TR on DNA Binding**—The C-TR contains several basic and hydrophobic amino acid residues (Fig. 1B). Provided that the region folds into α-helix, the basic residues appear to form a ridge on one side of the helix, whereas the hydrophobic residues appear on the other side as diagrammed in Fig. 3. We performed mutational analysis of the basic and hydrophobic residues in the C-TR to define the amino acid residues that are required for DNA binding. The DNA binding affinity of a series of MBP-Rme1p<sub>171–300</sub> C-TR mutant proteins was determined as described above. Fig. 4 shows gel mobility shift assays for each protein at the highest [protein]/[DNA] ratio examined, corresponding to lane 10 in Fig. 2, A–C.

We first tested the requirements for basic C-TR residues. Neutral substitutions for four basic residues (K290A/R291S/K293A/K294A) abolished detectable DNA binding (Fig. 4, lane 2). Individual K290A and R291A substitutions severely reduced binding affinity (lanes 4 and 5, respectively), whereas a K293A/K294S double substitution had no effect (lane 6). An R287A substitution also caused significantly less binding affinity (lane 3). A K300A substitution had no effect (lane 7). Therefore, basic residues Arg-287, Lys-290, and Arg-291 are critical for DNA binding, whereas Lys-293, Lys-294, and Lys-300 are not. The three critical residues (Arg-287, Lys-290, and Arg-291) are spatial neighbors in the helical C-TR projection (Fig. 3).

Next, we tested the requirements for hydrophobic C-TR residues. Mutant proteins with either F288A/L292S or I295A/L296S double substitutions did not bind to DNA (Fig. 4, lanes 8 and 9). We did not construct individual substitutions for each residue. Nonetheless, our results support the hypothesis that the hydrophobic surface predicted by the helical C-TR projection (Fig. 3) is required for DNA binding.

If the C-TR segment extending from Arg-287 to Leu-296 forms an α-helical structure, then helix-breaking proline substitutions in this region should abolish DNA binding. We observed that the side chains of Asn-289 and Lys-293 were not critical for DNA binding, because a N289A/K293A double substitution mutant protein bound to DNA with affinity similar to wild type (Fig. 4, lane 10). However, substitutions of proline for these residues abolished DNA binding (lane 11). These results
support the hypothesis that formation of an α-helix by this C-TR segment is required for DNA binding.

**Activity of C-TR Mutant Proteins in Vivo**—Our studies described above establish that the C-TR is required for DNA binding in vitro by recombinant MBP-Rme1p. To test the function of the C-TR in vivo, we examined the biological activity of mutant RME1 derivatives expressed in *S. cerevisiae*. The C-TR mutations were introduced into the *PGAL1-RME1* locus by recombination in strain AMP1122 (Fig. 5A; also see “Experimental Procedures”). We then used immunoblot analysis to verify...
that each Rme1p derivative was expressed, making use of the N-terminal SS3 epitope (13, 15) encoded by \( P_{GAL1} \)-RME1 (Fig. 5B). SS3 tagged-Rme1p was identified by its presence in a \( P_{GAL1} \)-RME1 strain (lane 1, AMP1122) and not in an rme1Δ strain (lane 2, AMP119). The wild-type Rme1p introduced by recombination from the \( malE \)-RME1 plasmid (lane 3, strain MHS11) was expressed at lower levels than in the \( P_{GAL1} \)-RME1 reference (lane 1), most likely because of the lack of a defined transcriptional terminator in 3' vector sequences (see Fig. 5A).

The mutant Rme1p derivatives, carrying either the K290A/R291S/K293A/K294A substitutions (Fig. 5B, lane 4, strain MHS13) or the K290A substitution (Fig. 5B, lane 5, strain MHS15), accumulated to levels as great as wild-type Rme1p (Fig. 5B, lane 3). A cross-reacting protein (indicated by an asterisk in Fig. 5B) was observed at comparable levels in all samples, thus verifying that similar amounts of extract protein were present in each sample. Therefore, these Rme1p C-TR substitutions do not cause diminished Rme1p accumulation.

We used two assays for Rme1p activity to assess mutant protein function. First, we examined repression of a hybrid \( RC-CYC1-lacZ \) reporter gene (14–16, 19). This reporter carries an \( IME1 \) upstream segment (−1746 to −2146) called a repression cassette (RC) adjacent to a \( CYC1-lacZ \) reporter gene. In control measurements, we found that wild-type Rme1p caused 14-fold repression (Fig. 6A, strain AMP1122) compared with non-functional Rme1–213p (strain AMP1124), in agreement with previous studies (14–16, 19). The reporter was expressed at low levels in strain MHS11, thus indicating that Rme1p derived from the \( malE \)-RME1 plasmid is capable of significant repression. Repression was not as great as in strain AMP1122, presumably because of the lower expression of Rme1p in strain MHS11 (Fig. 5B). In contrast, as seen in Fig. 6A, the reporter was expressed at high levels in strains MHS13 and MHS15. These results indicate that the C-TR substitutions block repression by Rme1p as expected for substitutions that impair DNA binding. We observed reproducibly that strain MHS15 (K290A substitution) was capable of weak repression compared with strain MHS13 (K290A/R291S/K293A/K294A substitutions), suggesting that the K290A substitution permits some DNA binding activity in vivo.

As a second assay, we examined the effect of C-TR mutations on sporulation. Diploids homozygous for the respective \( P_{GAL1} \)-RME1 alleles were constructed from strains AMP1122, AMP1124, MHS11, MHS13, and MHS15, and sporulation was quantitated as shown in Fig. 6B. Control diploids derived from AMP1122 sporulated weakly (0.6%); control diploids derived from AMP1124 sporulated efficiently (91.2%). The diploid derived from strain MHS11 sporulated weakly (0.4%), thus verifying that Rme1p derived from the \( malE \)-RME1 plasmid is capable of significant repression. The diploid derived from strain MHS13 sporulated efficiently (91.3%), thus verifying that the K290A/R291S/K293A/K294A substitutions abolish Rme1p function. Unexpectedly, the diploid derived from strain MHS15 sporulated at a low level (4.0%), which is substantially less than observed with the AMP1122 and MHS13 diploids. Yet, the level of sporulation in the MHS15 diploid is substantially greater than the MHS11 diploid, which supports the idea that the K290A substitution causes a repression defect. Therefore, the K290A substitution impairs but does not abolish DNA binding activity in vivo.

**DISCUSSION**

Herein, we have identified the DNA binding domain of Rme1p as residues 171–300, which consists of three zinc fingers and a C-terminal region of 16 amino acids (C-TR). Point mutations in the C-TR revealed that several basic amino acid residues (Arg-287, Lys-290, and Arg-291) and hydrophobic residues (Phe-288, Leu-292, Ile-295, and Ile-296) play an essential role in DNA binding in vitro. Mapping of the critical C-TR residues on an α-helical wheel projection suggests that the C-TR forms an amphipathic α-helix. In support of this structure, we found that a double proline substitution in the C-TR abolished DNA binding. Thus, we suggest that the basic surface of C-TR may contact DNA, whereas the hydrophobic surface may stabilize the structure of the DNA binding domain.

We also found that the C-TR is essential for transcriptional repression and inhibition of spore formation by Rme1p. This finding supports the importance of the C-TR for DNA binding in vivo. This analysis also pointed to an unexpected difference between the two C-TR substitutions tested; the K290A substitution caused a milder defect than the K290A/R291S/K293A/K294A multi-site substitution. The difference was negligible in transcriptional repression assays but was striking in sporulation assays. These results can be explained with the assumption that \( RC-CYC1-lacZ \) repression is a less sensitive functional assay than sporulation. This assumption seems reasonable because: (i) \( RC-CYC1-lacZ \) is a multi-copy reporter gene, whereas sporulation reflects mainly repression of the single-copy \( IME1 \) gene (14); and (ii) we have observed that \( RC-CYC1-lacZ \) repression depends upon overexpression of Rme1p, whereas sporulation inhibition occurs at the natural
Table I

Sequence alignments of C-terminal regions of zinc finger proteins with Rme1p C-TR homology

| Residue sequences | Proteins |
|-------------------|----------|
| HNNADSEFKRLKTIKNTK | RME1 C-TR |
| HTNSHPGCKGEEKRK | MIG1 (S. cerevisiae) |
| HTGOSQRELEK | MIG2 (S. cerevisiae) |
| HL-RHINTPSKRRK | Zinc finger protein (Candida albicans) |
| HNNPNSRNSHAAQL | CREA (Aspergillus aculeatus) |
| HNNPNSRNSHAKQK | Carbonic anhydrase (Ascomycota) |
| HNNPNSRNSNKKQK | Carbonic anhydrase (Sclerotium sclerotiorum) |
| HNVQNPPTNKK | Zinc finger protein (Ascobolus immersus) |
| HPGCDPRLNVTG | Zinc finger protein (putative) (Schizosaccharomyces pombe) |

a Sequence alignments start at the last histidine residue in the C-H2 zinc finger motif. The basic amino acids are underlined.

Fig. 7. Model for interaction of Rme1p with a DNA. Amino acid residues at positions −1, 2, 3, and 6 in the recognition α-helix of each finger are presented by their one-letter codes. Solid arrows depict the side chains of amino acids contacting the DNA sequence by its one-letter codes.

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