Repression by the yeast meiotic inhibitor RME1

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The *RME1* gene product, a negative regulator of meiosis with three zinc finger motifs, acts by preventing transcript accumulation from *IME1*, whose product is required for meiotic gene expression. We have isolated a 404-bp segment from a region 2 kb upstream of *IME1* that is sufficient for RME1-dependent repression of a heterologous promoter. This DNA contains an RME1-response element (RRE) and another region called the modulation region. The modulation region is required for repression because DNA containing the RRE alone did not repress but was able to confer RME1-dependent transcriptional activation of a reporter gene. In gel mobility retardation assays, RME1 formed a specific complex with the RRE, and RRE point mutations that reduced the affinity for RME1 also blocked repression and activation. Footprinting of the RME1–RRE complex revealed a 21-bp protected region that included the positions of these RRE mutations. We conclude that RME1 binding to this RRE is required for repression. Thus, the mechanism of meiotic inhibition by RME1 is direct transcriptional repression of *IME1*.

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Transcriptional repressors that are sequence-specific DNA-binding proteins can be distinguished by the position or context of their binding sites. For example, $\alpha 2$ –MCM1, $\alpha 1$ – $\alpha 2$, and ROX1 of yeast repress promoters lying within 300 bp of their binding sites (Johnson and Herskowitz 1985; Goutte and Johnson 1988; Keleher et al. 1988; Herskowitz 1989; Zitomer and Lowry 1992). These proteins may repress by recruiting a global negative regulatory complex, such as SSN6–TUP1 (Zhang et al. 1991; Keleher et al. 1992) or by positioning nucleosomes such that essential promoter sites are occluded (Shimizu et al. 1991; Roth et al. 1992). Other proteins, such as RAP1 of yeast, have different properties. RAP1 acts as a positive regulator through an isolated binding site (Brand et al. 1987; Shore and Nasmyth 1987). When the binding site is within a region called a silencer, however, RAP1 acts to repress target promoters >1000 bp away (Shore and Nasmyth 1987; Sussel and Shore 1991). Repression appears to require that RAP1 and other silencer-binding factors, such as ABF-1, establish or maintain an altered chromatin state that is inaccessible to RNA polymerases, endonucleases, and methylases (Nasmyth 1982; Strathern et al. 1982; Schnell and Rine 1986; Shore and Nasmyth 1987; Shore et al. 1987; Buchman et al. 1988; Kayne et al. 1988; Singh and Klar 1992).

Our studies focus on the yeast RME1 protein, a zinc finger protein homolog that inhibits meiosis and spore formation (Kassir and Simchen 1976; Mitchell and Her-

skowitz 1986; Covitz et al. 1991). *RME1* is expressed in **a** and α cells, which are thus unable to enter meiosis, and is repressed in **a**/ α cells, which can enter meiosis in response to starvation (Mitchell and Herskowitz 1986). Genetic and molecular studies indicate that RME1 exerts its effects by blocking transcript accumulation from the *IME1* gene (Kassir et al. 1988; Covitz et al. 1991). The *IME1* product is required for expression of *IME2* and many other meiotic genes (Engbrecht and Roeder 1990; Mitchell et al. 1990; Smith et al. 1990). There are several positive regulators of *IME1* that, in principle, might be more direct targets of repression by RME1 (Neugeborn and Mitchell 1991; Shah and Clancy 1992; Su and Mitchell 1993). So far, none of those genes appears to be regulated by RME1. These findings are consistent with the notion that RME1 represses *IME1* more directly.

Here, we report that RME1 binds specifically to a site 2 kb upstream of the *IME1* gene and that the RME1 binding site is required for full repression of *IME1*. RME1 can either repress or activate transcription of a reporter gene, depending on the context of its binding site. Our results indicate that RME1 is a new example of a transcription factor capable of effecting either negative or positive regulation. Further, the context of the RME1-binding site is key in determining which activity will be manifested.

Results

Isolation of an RME1-dependent repression cassette

To identify a region involved in cell-type control of *IME1*

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expression, we performed a deletion analysis of a plasmid-borne *IME1* gene. We compared sporulation in *a/a* and *a/α* diploids to monitor the response of plasmid deletion derivatives to cell type signals (Fig. 1). *a/a* diploids are unable to sporulate in part because they express *RME1*, which in turn represses *IME1*. We reasoned that a properly regulated *IME1* gene would not stimulate sporulation of *a/a* strains but that deletions permitting *IME1* expression would result in higher levels of sporulation. As a control, we tested the ability of each plasmid to stimulate sporulation in an *a/α ime1Δ/ime1Δ* strain. Sporulation of this strain confirmed that each *IME1* derivative was expressed in the absence of *RME1*, which is transcriptionally repressed in *a/α* cells. Plasmids containing 5' deletion end points between -5600 and -2243 appeared to be properly regulated by cell-type signals: They permitted much lower levels of sporulation in the *a/a* diploid than in the *a/α* control strain (Fig. 1, pAC55-2, pAC58, pAC67, pAC70, and pAC82; the basal level of sporulation of the *a/a* strain bearing these plasmids may reflect the increased *IME1* gene dosage). Plasmids with deletion end points from -2001 to -1202 , however, had elevated levels of *IME1* expression in the *a/a* strain: They stimulated more similar levels of sporulation in the *a/a* and *a/α* diploids (Fig. 1, pAC85-26, pAC62, and pAC59). An internal deletion that eliminated sequences from -1954 to -1203 also permitted elevated levels of *IME1* expression in the *a/a* strain (Fig.

1, pAC87-2), whereas a deletion from -1742 to -1119 did not (Fig. 1, pAC164). We delineated the 5' border of the region that influences *IME1* expression, -2243 , by comparing the end points of pAC82 and pAC85-26. The 3' border, -1743 , was delineated by comparing the end points of pAC87-2 and pAC164.

The deletions in this 501 bp upstream region may have either eliminated a *cis*-acting regulatory sequence or affected a gene encoding a *trans*-acting factor. The ability of the deletion derivatives to stimulate sporulation of either the *a/a* test strain or the *a/α* control strain required the presence of an intact *IME1*-coding sequence (Fig. 1, pAC72 and pAC73). In addition, we found no long open reading frame between -2243 and the *IME1*-coding region (≤ 74 codons), indicating that there is no neighboring protein-coding sequence that might be affected. We conclude that the 501 bp upstream regulatory region from -2243 to -1743 acts in *cis* to *IME1*.

Deletions of the upstream regulatory region may have abolished repression by a cell-type control pathway. Alternatively, such deletions may have caused a general increase in *IME1* expression. We distinguished between these two possibilities by direct measurement of *IME1* RNA expression from pAC82, which contains the upstream regulatory region, and pAC85-26, which has a deletion in this region (see Fig. 1). To focus on the effects of *RME1* alone, we compared *IME1* transcript levels in *a/α ime1/ime1* strains that differed in their expression of

Figure 1. Sporulation assay for plasmid *IME1* expression. *a/a* and *a/α ime1/ime1* diploids bearing each plasmid were incubated on sporulation medium for 3 days, and the percent sporulation was determined. The hatched box represents the upstream regulatory region. Deletion end points are numbered with respect to the *IME1* initiation codon (+1). The *IME1* RNA start sites lie between -210 and -280 (Smith et al. 1990; Sherman et al. 1993). End points in parentheses are approximate. The strains used were 931 and 805/808. Restriction sites are (B) *Bam*HI; (Sa) *Sau*3A; (X) *Xho*I; (G) *Bgl*II; (N) *Nhe*I; (M) *Msc*I; (K) *Kpn*I; (Sp) *Sph*I.

Figure 2. Effect of RME1 on *IME1* expression from plasmid deletion derivatives. RNA was prepared 0, 2, or 4 hr after starvation from α/α strains bearing the *IME1* plasmid deletion derivative pAC82 (lanes 1–6) or pAC85-26 (lanes 7–12). RME1 expression was regulated from P_{GAL1} : RME1 not expressed [(–) lanes 1–3 and 7–9], or expressed [(+) lanes 4–6 and 10–12]. The strains were 1049/1053 and 1050/1051.

RME1 from the *GAL1* promoter (Fig. 2). The P_{GAL1} -*RME1* gene was regulated independently of cell type by use of wild-type or null alleles of *GAL80*, a negative regulator of the *GAL1* promoter. *IME1* expression from both pAC82 and pAC85-26 increased upon starvation in cells lacking RME1 (Fig. 2, lanes 1–3, 7–9). RME1 blocked this increase from pAC82 (Fig. 2, cf. lanes 1–3 and 4–6). In contrast, RME1 had no effect on *IME1* expression from pAC85-26 (Fig. 2, cf. lanes 7–9 and 10–12). We conclude that the upstream regulatory region is required for repression of the plasmid-borne *IME1* gene by RME1. This region does not appear to have a role in the nutritional control of *IME1* transcription.

To determine whether this region is sufficient for RME1-dependent repression, we examined its effect on the heterologous *CYC1* promoter. [The separable upstream activating sequences (UASs) in the *CYC1* promoter (Guarente and Mason 1983) are here referred to

collectively as UAS_{*CYC1*}.] We placed the 501-bp regulatory segment (–2243 to –1743) upstream of UAS_{*CYC1*} in the *cyc1-lacZ* reporter plasmid pKB112 and measured β -galactosidase activity in a strain expressing RME1 and in a control strain expressing RME1-213, a nonfunctional missense derivative (Covitz et al. 1991). The upstream regulatory region repressed UAS_{*CYC1*} 17-fold in the strain expressing RME1 but not in the control strain (Fig. 3, pAC137-4). This value is in good agreement with estimates of 6- to 30-fold repression of chromosomal *IME1* RNA levels by RME1 (Kassir et al. 1988; Smith and Mitchell 1989). We defined more narrowly the DNA responsible for RME1-dependent repression by constructing deletion derivatives and assaying RME1-dependent repression of UAS_{*CYC1*} (Fig. 3). A deletion that left the 404 bp from –2146 to –1743 was sufficient to give 27-fold repression (Fig. 3, pAC153-4). A fragment containing an internal deletion from –2016 to –1976 still gave over sixfold repression (Fig. 3, pAC157-2). The remaining sequences, –2146 to –2017 and –1975 to –1743, were required for repression (Fig. 3, pAC160-2, pAC152-3, pAC141-10, and pAC142-10). These data establish that the 404 bp fragment from –2146 to –1743 is sufficient for repression by RME1. The sequence of this DNA, which we call the repression cassette, is shown in Figure 4.

Identification of an RME1-response element

The above analyses and further experimentation indicated that the repression cassette can be divided into two distinct segments. Each is required for RME1-dependent repression. The first segment, –2146 to –2017, contains an RME1-response element (RRE), the evidence for which is described below. The other segment, –1975 to –1743, is referred to as the modulation region. A chance observation enabled us to identify the segment containing the RRE. We noticed that the repression cassette stimulated gene expression when transferred into the

Figure 3. RME1-dependent repression of the *CYC1* promoter. pKB112 contains UAS_{*CYC1*} fused to a *lacZ* reporter gene (not to scale). Derivatives contain insertions from the *IME1* upstream regulatory region; end points are numbered with respect to the *IME1* initiation codon. Strains that expressed RME1 or RME1-213 and contained these plasmids were assayed for β -galactosidase activity. The hatched box indicates the RRE; the stippled box indicates the modulation region. The strains were 1122 and 1124.

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-2146 GGTGTTGGAGTAATTTGATTAATGTATTCCCTCACGGAGGTTTCAGTAGATATTCTCAATAAAAGAAGGGTGGGGGTGTAT
                                     A
                                     ↑
-2066 TTGCTGCAAGAATGCGGATGTCAAAAGAACCTCAAGAAGTCCACTAAATGGCAGTAAATGCCGCGCTTACATTTTACGG
                                     ↓ ↓ ↓
                                     G GG

-1986 ACTGCCGAAAACGTACGGCTAACTGCTGTACCTCAAAAGCATAAAATTGTGATGTTGATTAAACAAGTAGGCGTTGCAT
-1906 ATATAGACGTGGGTAGGAAAAAGTGAGCGCCAACACTATATAAGAAACCGTTTCTGGATACACCCTCGTGAAGAAATAG
-1826 GGTCTGCATTGATATTTTCAAACCTTATATAATTAATAATAATTAATAGCGCTTAGTTTAAAGAATTTGAACATTTTTT
-1746 GGCC

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Figure 4. Sequence of the repression cassette. The RRE ↓ mutations are indicated below their position, the RRE ↑ mutation is indicated above its position (see Tables 1 and 2). –2044 to –2024, the region in the RME1–DNA complex protected from chemical nuclease scission, is shown in boldface type (see Fig. 8).

cyc1-lacZ reporter plasmid pLGΔ312SΔSS, which lacks UAS_{CYC1} , and that activation was RME1 dependent (Fig. 5, cf. pLGΔ312SΔSS and pAC155-2). Expression was 5- to 10-fold higher when the modulation region was removed (Fig. 5, cf. pAC155-2 with pAC106-4 and pAC110-6). Further deletions indicated that the 130 bp from –2146 to –2017 contained the DNA required for RME1-dependent activation (Fig. 5, pAC110-6), suggesting that an RRE lay within this sequence. The region from –2146 to –2017 alone was sufficient for activation (Fig. 5), but both this segment and the modulation region were required for repression (Fig. 3).

We conducted a mutational analysis to identify specific base pairs involved in RME1-dependent activation. Mutant fragments containing the sequence from –2146 to –2017 were generated and transferred into a *cyc1-lacZ* reporter vector that lacked UAS_{CYC1} (see Materials and methods). This analysis identified two classes of RRE mutations. The first includes three mutations that eliminated RME1-dependent activation (Table 1). These were designated RRE ↓ 1, RRE ↓ 2, and RRE ↓ 3 and corresponded to the base changes –2035^{T→G}, –2033^{A→G}, and –2032^{A→G}, respectively (Table 1). The second class contains one member that enhanced RME1-dependent β-galactosidase expression 100-fold (Table 1). This mutation was designated RRE ↑ 4, and corresponded to the base change –2031^{G→A}.

The phenotypes of the RRE mutations in the RME1-dependent activation assay suggested that we had identified an authentic RRE. It was not clear, however,

whether these base pairs were also involved in RME1-dependent repression of *IME1*. We replaced wild-type sequences with the RRE mutations upstream of the *IME1* gene on plasmids to examine their effect on repression. *IME1* expression was monitored by comparing sporulation in *a/a* and *a/α ime1/ime1* strains. The three RRE ↓ mutations each permitted increased levels of sporulation in the *a/a* diploid (Table 2, pAC136, pAC118-1, and pAC118-2). Thus, the RRE ↓ mutations cause elevated expression of the plasmid-borne *IME1* gene (loss of repression) in the *a/a* strain. The RRE ↑ mutation did not permit increased levels of sporulation in the *a/a* diploid (Table 2, pAC118-3), indicating that repression was maintained. We conclude that these mutations lie in an RRE that is important for both RME1-dependent activation of the *cyc1-lacZ* reporter gene and RME1-dependent repression of *IME1*.

Binding of RME1 to RRE-containing DNA

We used a gel mobility retardation assay to test whether RME1 binds to DNA that contains the RRE. The probe was *IME1* –2146 to –2017, the segment that caused RME1-dependent activation of the *cyc1-lacZ* reporter. One major complex was identified with an extract that contained RME1 (Fig. 6, lane 3). It was absent with extracts from *rme1Δ* or *rme1-213* strains (Fig. 6, lanes 2 and 4, respectively). We used an extract containing RME1Δ2-117, a functional, truncated derivative to determine whether RME1 itself was present in the complex (Hope

Figure 5. RME1-dependent activation. pLGΔ312SΔSS contains a *cyc1-lacZ* gene that lacks UAS_{CYC1} (not to scale). Derivatives contain insertions from the repression cassette. Strains, assays, and symbols were as described in the legend to Fig. 3.

Table 1. Effects of RRE point mutations on RME1-dependent activation

Plasmid ^a	Mutation (name)	β-Galactosidase units ^b	
		RME1	RME1-213
pAC119-10	none (RRE)	7.6 ^c	0.5
pAC128-4	–2035 ^{T→G} (RRE ↓ 1)	0.7	0.5
pAC119-1	–2033 ^{A→G} (RRE ↓ 2)	0.5	0.5
pAC119-2	–2032 ^{A→G} (RRE ↓ 3)	0.7	0.7
pAC119-3	–2031 ^{G→A} (RRE ↑ 4)	730	0.5

^aConstructs replaced UAS_{CYC1} in pKB112 with an *IME1* –2146 to –2017 segment containing the indicated mutation.

^bStrains and assays were as described in the legend to Fig. 3.

^cDifferent absolute levels of RME1-dependent β-galactosidase activity were observed here compared with insertions in pLGΔ312SΔSS (cf. pAC119-10 here with pAC110-6 in Fig. 5). The difference is likely attributable to the different polylinker sequences flanking the insertions.

and Struhl 1986). The truncated derivative formed a complex with greater mobility than that of the complex containing the full-length protein (Fig. 6, cf. lanes 3 and 6). An extract from a strain expressing both the full-length and the truncated polypeptides yielded both complexes (Fig. 6, lane 5). These data indicate that RME1 binds to the –2146 to –2017 segment.

To determine the specificity of the RME1–DNA complex, we used a gel mobility retardation competition assay. Each binding reaction included wild-type labeled probe DNA (–2146 to –2017) and various unlabeled competitor DNAs. DNA containing the RRE ↓ 1, RRE ↓ 2, or RRE ↓ 3 mutation competed poorly compared with DNA containing the wild-type RRE (Fig. 7, lanes 3–7, 10–16). DNA containing the RRE ↑ 4 mutation competed more efficiently than DNA containing the wild-type sequence (Fig. 7, lanes 10–12, 17, 18; data not shown). These experiments demonstrate a correlation between the effects of the RRE mutations on the response to RME1 in vivo and on RME1–DNA complex formation in vitro: The RRE ↓ mutations block activation of the *cyc1*–*lacZ* reporter gene (Table 1), impair repression of *IME1* (Table 2), and reduce the binding affinity for RME1 protein (Fig. 7); the RRE ↑ mutation enhances activation of the *cyc1*–*lacZ* reporter and increases the affinity for RME1 protein.

Table 2. Effects of RRE point mutations on repression of *IME1*

Plasmid ^a	Mutation (name)	Percent sporulation ^b	
		a/a	a/α
pAC82	none (RRE)	0.8	49
pAC136	–2035 ^{T→G} (RRE ↓ 1)	11	52
pAC118-1	–2033 ^{A→G} (RRE ↓ 2)	25	66
pAC118-2	–2032 ^{A→G} (RRE ↓ 3)	15	43
pAC118-3	–2031 ^{G→A} (RRE ↑ 4)	1	45

^aPlasmids contained the indicated mutation upstream of *IME1*.

^bStrains and assays were as described in the legend to Fig. 1.

Figure 6. Gel mobility retardation assays with labeled *IME1* –2146 to –2017 DNA and crude RME1 extracts. Probe was mixed with extract and then electrophoresed on a native 5% polyacrylamide gel. The RME1 derivatives present in each extract are indicated above the lane numbers. The upper arrow indicates the mobility of the RME1–DNA complex; the lower arrow indicates the mobility of the RME1Δ2-117–DNA complex. Extracts were from strains 930, 1043, 1073, 1174, and 1175.

Further delineation of the RRE

We defined more precisely the DNA responsible for binding RME1 by use of chemical nuclease footprinting. The probe contained *IME1* –2146 to –2017 and included the RRE ↑ 4 mutation. This mutation increased the proportion of probe that was in the RME1–DNA complex and thus facilitated isolation of the complex for footprinting. We observed a 21-bp protected region corresponding to nucleotides –2044 to –2024 of *IME1* (Fig. 8). This sequence includes the base pairs changed in all four of the RRE mutations (Fig. 4). We conclude that this 21-bp segment is included in the RME1-binding site.

To confirm that the DNA defined by nuclease protection is the RRE responsible for activity in vivo, we tested its ability to cause RME1-dependent repression of the *CYC1* promoter. A synthetic oligonucleotide that contained the wild-type sequence from –2046 to –2022 (the protected region plus 2 flanking base pairs on each side) was inserted next to the modulation region upstream of UAS_{CYC1}. This construct was able to repress *cyc1*–*lacZ* expression fivefold when RME1 was expressed, whereas a construct that lacked the oligonucleotide insertion was unable to repress (Fig. 3, cf. pAC160-2 and pAC170-6). Insertion of two RREs gave a similar level of repression to that found with one element inserted (Fig. 3, pAC169-1 and pAC170-6). Thus this 25-bp sequence, in conjunction with the modulation

Figure 7. Competition of labeled RME1–DNA complex formation with unlabeled DNA fragments. The labeled probe was *IME1* –2146 to –2017. Binding reactions contained no extract (lanes 1,8); *rme1Δ* extract (lanes 2,9); RME1 extract (lanes 3–7,10–18). Excess unlabeled competitor [250-fold (lanes 4,6,11,13,15,17) or 500-fold (lanes 5,7,12,14,16,18)] was added to the binding reactions. Competitors were *IME1* –2146 to –2017 with wild-type (lanes 4–5,11–12) or mutant (lanes 6–7,13–18) RREs, as indicated above the lane numbers. The arrow indicates the mobility of the RME1–DNA complex. Extracts were from strains 930 and 1043. Lanes 1–7 and 8–18 represent separate experiments.

region, is sufficient for RME1-dependent repression and contains a complete RRE.

All of our experiments thus far had been conducted on plasmids. To test the function of the RRE in its natural location, we examined the consequences of a chromosomal RRE deletion. The allele *IME1-81* lacks the base pairs from –2044 to –2025. We compared *IME1*⁺ and *IME1-81* expression in α/α diploids that differed in their expression of *RME1* from the *GAL1* promoter. Measurement of *ime2-lacZ* expression and sporulation efficiency was used as assays (Table 3). Control strains that did not express RME1 gave high levels of β -galactosidase activity (508–619 units) and sporulation (82–90%) when ho-

mozygous for either the *IME1*⁺ or the *IME1-81* allele. A strain that expressed RME1 and was homozygous for the *IME1*⁺ allele gave only 2.7 β -galactosidase units and 1.3% sporulation. A strain that expressed RME1 but was homozygous for the *IME1-81* allele, however, produced 45 units of β -galactosidase activity and sporulated to 51% efficiency. We conclude that the deletion in *IME1-81* removed sequences necessary for complete repression of the chromosomal *IME1* gene by RME1.

Discussion

Our present studies provide the first direct information concerning the mechanism of RME1 action. We have shown that RME1 binds to a site upstream of *IME1* that is required for repression and, furthermore, that the RME1-binding site and flanking region is sufficient to confer RME1-dependent repression on a heterologous gene. These studies have extended our understanding of how the mating-type locus governs expression of meiotic genes: $\alpha 1$ – $\alpha 2$ represses *RME1* directly (Goutte and Johnson 1988; Covitz et al. 1991), and RME1 represses *IME1* directly.

Nature of the RRE

The RRE is defined by its properties in vivo: It can support RME1-dependent activation of a reporter gene or (with the modulation region) repression of a heterologous promoter. It is required for repression of a plasmid-borne *IME1* gene, and it is essential for full RME1-de-

Figure 8. Chemical nuclease footprint of the RME1–DNA complex. The probe included *IME1* –2146 to –2017 containing the RRE $\uparrow 4$ mutation and was labeled on the top strand. A preparative gel mobility retardation was used to separate free and bound probe. Gel slices were excised and treated with 1,10-phenanthroline–copper ion reagent as described in Materials and Methods. DNA was recovered and electrophoresed on an 8% sequencing gel. Maxam and Gilbert G and G + A reactions were run as markers. The indicated nucleotide sequence is *IME1* –2044 to –2024 with the RRE $\uparrow 4$ mutation.

Table 3. Chromosomal deletion of the RRE

<i>IME1</i> genotype ^a	RME1 expression	β -gal units ^b		Percent sporulation
		veg	spo	
+ / +	–	5	508	90
81/81	–	3.9	619	82
+ / +	+	0.6	2.7	1.3
81/81	+	0.7	45	51

^aStrains were 1285/1286, 1283/1282, 1285/1287, and 1283/1284.

^bValues represent *ime2-lacZ* expression.

pendent repression of the chromosomal *IME1* gene. Our biochemical assays show that the RRE includes an RME1-binding site that spans 21 bp.

Studies of the zinc finger proteins SP1 and Zif268 (Nardelli et al. 1991; Pavletich and Pabo 1991) suggest that a protein like RME1, with three zinc fingers, should bind to a 9-bp site. Why is the protected region in the RME1-dependent complex roughly twice this size? One explanation is that RME1 structural features lead to more extensive DNA contacts than for these other proteins. For example, the nonconsensus spacing between RME1 zinc fingers (Covitz et al. 1991) may result in a more elongated structure. A second explanation is that RME1 is a subunit of an oligomeric DNA-binding protein. We note that the protected region includes most of an imperfect direct repeat of the sequence TCAA^(A/G)A^(G/A)A^(A/G)CC, which we refer to as the RRE half-site. All three RRE ↓ mutations alter base pairs conserved by the two putative half-sites. In contrast, the RRE ↑ 4 mutation, which improves binding, makes the downstream half-site more similar to the upstream half-site. These mutational changes are consistent with the idea that the same polypeptide recognizes both half-sites. Our estimate of the size of RME1 in solution in crude extracts, based on gel filtration studies, is 70 kD, the size expected for a homodimer (P.A. Covitz, unpubl.). If RME1 bound as a homodimer, however, we would expect to find a novel gel mobility retardation complex in extracts containing both RME1 and RME1Δ2-117, representing a complex of the RRE, RME1, and RME1Δ2-117. The absence of this complex suggests that RME1 and RME1Δ2-117 do not dimerize. This finding may indicate that RME1Δ2-117 has an impaired ability to dimerize. Alternatively, RME1 may not bind as a homodimer but, rather, as part of a heteromeric complex. Experiments with purified RME1 should help to resolve this issue.

Transcriptional activation by RME1

We observed that RME1 can activate transcription from the RRE. Activation and repression by RME1 may reflect alternate states or complexes, because activation is most efficient in the absence of the modulation region, whereas repression requires the modulation region. The ability of RME1 to activate may result from the similarity of its amino terminus to an acidic activation domain (net charge of −7 for residues 1–100).

There are two indications that activation requires relatively efficient formation of the RME1–RRE complex. First, we have observed activation only when RME1 is overexpressed (from the *P_{GAL1}–RME1* gene). Second, activation is improved 100-fold by the RRE ↑ 4 mutation, which increases the efficiency of RME1–RRE complex formation in vitro. It is unlikely that the RRE ↑ 4 mutation causes a qualitative change in the RME1–RRE complex because it is capable of directing repression of *IME1*. Experiments with *Drosophila* Krüppel have shown that its expression levels alone can determine whether it activates (high expression) or represses (low expression) (Sauer and Jäckle 1991). RME1 apparently has different

properties, because its high-level expression permits both repression and activation. Thus, the ability of RME1 to activate or repress is determined by its expression levels as well as the context of the RRE.

These findings raise the question of whether RME1-dependent activation has any biological significance. *IME1* is activated only in a/α cells, whereas *RME1* is repressed in this cell type (Mitchell and Herskowitz 1986; Smith and Mitchell 1989). Furthermore, the RRE is not involved in the activation of *IME1* by nutritional signals (see Fig. 2 and Table 3). Therefore, neither RME1 nor its binding site has a role in the activation of *IME1*. *RME1* RNA levels, however, increase 10-fold after starvation in a or α haploid cells (P.A. Covitz, unpubl.). Thus, RME1 might activate a target gene not yet identified in starved haploid cells.

The modulation region

The 404-bp RME1 repression cassette includes functionally distinct upstream and downstream segments. The upstream segment (−2146 to −2017) is necessary only to provide the RRE, because it may be functionally replaced by a synthetic RRE. The precise function of the downstream segment (−1975 to −1743), the modulation region, is less clear. Deletions of either end of the modulation region destroy repression, suggesting either that there are multiple functional sites within the element or that precise spacing between the RRE and a second site is critical for repression. We note that the modulation region has potential binding sites for the silencer-binding proteins ABF1 (−1874 to −1859) and RAP1 (−1855 to −1847); each of these sites has a 1-bp deviation from the respective consensus (Ghosh 1992). Neither of these sites, however, is removed in any of the several deletions that abolish repression of *UAS_{CYC1}*. Clearly, a more systematic mutational analysis of the modulation region will be required to understand its functional requirements.

Redundancy of RME1-dependent repression sites

We have shown that DNA containing an RME1-binding site is essential for RME1-dependent repression of *UAS_{CYC1}* by the repression cassette. This site, −2044 to −2024, also contributes to repression of the chromosomal *IME1* gene. Both assays of *IME1* expression—sporulation and *ime2–lacZ* levels—however, indicate that the deletion allele *IME1-81*, which lacks the RRE, is still partially repressed by RME1. One explanation might be that RME1 has some effect on these assays that is independent of *IME1*. We found previously, however, that expression of *IME1* from the *GAL1* promoter renders sporulation and *ime2–lacZ* expression insensitive to RME1 (Covitz et al. 1991). Therefore, the incomplete constitutivity of the *IME1-81* mutation must result from direct effects on *IME1* expression. These observations suggest that RME1 is able to interact with the *IME1* regulatory region through sites other than the −2044 to −2024 RRE.

Where might additional RME1-binding sites lie? Existence of a far upstream site, near -3000 , was deduced from a previous study of fragments flanking *IME1* that could titrate RME1 activity when present on multicopy plasmids (Granot et al. 1989). This putative site is not essential for repression, though, because we found that deletion of all sequences upstream of -2243 from a plasmid-borne *IME1* gene did not relieve repression (Fig. 1, cf. pAC55-2 and pAC82). On the basis of homology to the RRE half-site, we found a putative RME1-binding site within the *IME1*-coding region ($+111$ to $+121$). Similarly, this putative half-site is not essential for repression because its deletion from the chromosome does not relieve repression (P.A. Covitz, unpubl.). These findings are consistent with the idea that there are multiple RME1-binding sites near *IME1* with partially overlapping functions.

Size of the *IME1* regulatory region

Most yeast genes have regulatory regions that extend 200–500 bp upstream of the coding region. Why should the *IME1* regulatory region be so large? We believe that properties of the *HO* gene, which has a 1.5-kb regulatory region, may provide some insight. The *HO* gene is regulated by multiple signals. Dissection of the *HO* upstream region has revealed separable sites that confer repression by $\alpha 1$ – $\alpha 2$, dependence on SWI4–SWI6, and restriction of expression to mother cells (for review, see Herskowitz 1989). *IME1* also responds to several regulatory signals. *IME1* expression depends on at least two pathways, one involving *MCK1* and the other involving *RIM1* (Neugeborn and Mitchell 1991; Su and Mitchell 1993). In addition, cAMP limitation causes elevated *IME1* expression (Smith and Mitchell 1989; Matsuura et al. 1990) and appears to act independently of *MCK1* and *RIM1* (L. Neugeborn, pers. comm.). Regulation of *IME1* by the mating-type locus also depends on at least two pathways, one involving *RME1* and the other involving *IME4* (Shah and Clancy 1992). Therefore, the long regulatory region of *IME1* may also be useful for coordinating multiple regulatory signals.

Materials and methods

Strains and RME1 alleles

Yeast strains were all isogenic to SK-1 (Kane and Roth 1974); genotypes are listed in Table 4. Growth media were as described previously (Smith and Mitchell 1989). Strains used for Northern analyses, β -galactosidase assays, and protein extracts expressed *RME1* derivatives from the *GAL1* promoter. The *P_{GAL1}-RME1* genes were integrated at one of two different loci: *URA3* (*ura3::P_{GAL1}-RME1::URA3*) or *RME1* (*rme1::P_{GAL1}-RME1::TRP1*). Integration at *URA3* was described previously (Covitz et al. 1991). The constructs at *RME1* were created by one-step replacement of the native gene (Rothstein 1983) with *P_{GAL1}-RME1* and sequences from the integrating vector pRS304 (Sikorski and Hieter 1989), which contains the *TRP1* marker. The *P_{GAL1}-RME1* derivatives used include *P_{GAL1}-S53-RME1*, which encodes a functional epitope-tagged derivative (Covitz et al. 1991); *P_{GAL1}-S53-rme1-213*, a nonfunctional mutant allele

Table 4. Yeast strains

Strain	Genotype ^a
805	a <i>arg6 his3 rme1-5::LEU2 ime1-13::HIS3 GAL80</i>
808	α <i>his3 ime1-13::HIS3 GAL80</i>
930	a <i>his4-G rme1-5::LEU2 pep4::URA3</i>
931	a/a <i>arg4/ARG4 his4-G/his4-N ade3/ADE3</i>
1043	a <i>his4-G rme1-5::LEU2 ura3::P_{GAL1}-S53-RME1::URA3 pep4::URA3</i>
1049	α <i>arg6 his3 rme1-5::LEU2 ime1-13::HIS3 ura3::P_{GAL1}-S53-RME1::URA3</i>
1050	a <i>arg6 his3 rme1-5::LEU2 ime1-13::HIS3 ura3::P_{GAL1}-S53-RME1::URA3</i>
1051	α <i>his3 rme1-5::LEU2 ime1-13::HIS3</i>
1053	a <i>his3 rme1-5::LEU2 ime1-13::HIS3 GAL80</i>
1073	a <i>his4-G rme1-5::LEU2 ura3::P_{GAL1}-S53-rme1-213::URA3 pep4::URA3</i>
1122	α <i>rme1::P_{GAL1}-S53-RME1::TRP1</i>
1124	α <i>rme1::P_{GAL1}-S53-rme1-213::TRP1</i>
1174	a <i>his4-G rme1::P_{GAL1}-S53-RME1::TRP1 ura3::P_{GAL1}-RME1Δ2-117:URA3 pep4::URA3</i>
1175	a <i>his4-G rme1-5::LEU2 ura3::P_{GAL1}-RME1Δ2-117:URA3 pep4::URA3</i>
1269	a <i>his4-G rme1-5::LEU2 ura3::P_{GAL1}-S53-RME1::URA3 pep4::URA3</i> [pAC34]
1282	a <i>IME1-81 GAL80</i>
1283	α <i>his4-G rme1::P_{GAL1}-S53-RME1::TRP1 IME1-81 ime2-4-lacZ::LEU2</i>
1284	a <i>IME1-81</i>
1285	a <i>ime2-4-lacZ::LEU2</i>
1286	α <i>his4-G rme1::P_{GAL1}-S53-RME1::TRP1 GAL80</i>
1287	α <i>his4-G rme1::P_{GAL1}-S53-RME1::TRP1</i>

^aAll strains have the additional alleles *leu2 trp1 ura3 lys2 ho::LYS2 gal80::LEU2*, except as indicated.

that encodes a Cys $\rightarrow$ Ser substitution at residue 213 in the second zinc finger motif (Covitz et al. 1991); and *P_{GAL1}-RME1 Δ 2-117*, described below.

P_{GAL1}-RME1 Δ 2-117 encodes a truncated derivative of RME1 that lacks residues 2–117 but retains the three carboxy-terminal zinc finger motifs. This gene was constructed by digesting plasmid pAM240 (Covitz et al. 1991) at the unique *HindIII* site in the vector and the *BglIII* site at codon 117 in *RME1*. The intervening DNA corresponding to the 5' end of the gene was eliminated, and the plasmid was recircularized with an oligonucleotide adapter containing *HindIII* and *BglIII* sticky ends. The adapter was made by annealing the oligonucleotides 5'-AGCT-TATGCCCCGGGGA-3' (top strand) and 5'-GATCTCCCCCGGGCATA-3' (bottom strand). The resulting construct (pAC Δ 2-117) contained codons for the residues Met, Pro, and Gly fused in-frame to codons 118–300 of *RME1*. This derivative was transferred on a *HindIII*-*XhoI* fragment into the *HindIII* and *SalI* sites of pBM272, which contains the *GAL1* promoter (Johnston and Davis 1984); the resulting plasmid was called pAC12. *P_{GAL1}-RME1 Δ 2-117* was transferred from pAC12 into Yip5 on an *EcoRI* partial digest fragment and integrated at *URA3* by digestion with *StuI* and transformation into yeast. Integration of a single copy was confirmed by Southern analysis. *P_{GAL1}-RME1 Δ 2-117* blocked expression of an *ime2-lacZ* fusion in haploid strains that contained a deletion of the native *RME1* gene. Therefore *P_{GAL1}-RME1 Δ 2-117* is a functional derivative.

All strains used for protein extraction contained a disruption in the *PEP4* protease gene, created by transformation of the

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pep4::URA3 disruption plasmid pTS15 (Rothman et al. 1986), digested with *EcoRI* and *XhoI*, and confirmed by Southern analysis.

IME1 isolation and deletions

Our original *IME1* clone was isolated through a selection for high-copy plasmids that bypassed *RME1* activity (Smith and Mitchell 1989). This strategy raised the concern that our clone may have acquired a mutation that relieved repression by *RME1*. To avoid such a complication, we used a nonselective screen to obtain plasmid pAC55-2, the source of *IME1* for the present studies. The *IME1* gene on pAC55-2 was isolated from the SK-1 yeast genetic background by colony hybridization in bacteria. An SK-1 library was generated from genomic DNA partially digested with *Sau3A* and ligated into the *Bam*HI site of the low-copy shuttle vector pRS314 (Sikorski and Hieter 1989). A 2.3 *Bgl*III fragment from pAM500 [which contains the previously isolated *IME1* gene (Smith and Mitchell 1989)] was used as a probe. DNA from a single positive colony was purified, and the plasmid was found to contain an 8.6-kb insert. Identity of the *IME1* gene on the insert was confirmed by (1) comparison of restriction digest patterns of pAC55-2 and pAM500; (2) partial sequencing of the *IME1*-coding region on pAC55-2; and (3) genetic complementation of an *ime1* deletion (see Fig. 1). The derivatives of pAC55-2 shown in Figure 1 were constructed by deleting DNA between restriction sites or by digesting with exonuclease III and S1 nucleases. The Sequenase–dideoxy method (U.S. Biochemicals) was used to sequence the top strand of *IME1* from –2243 to –1078. This provided 44 bp of overlap with the upstream end of the previously published sequence (Smith et al. 1990). The bottom strand was sequenced on an Applied Biosystems 373A automated sequencer.

Subclones of the upstream regulatory locus were generated by PCR amplification and ligated into the *Kpn*I site upstream of UAS_{CYC1} in pKB112 [a derivative of pLGΔ312S (Guarente and Mason 1983; Bowdish and Mitchell 1993)], the *Xho*I site upstream of pLGΔ312SΔSS (Guarente and Mason 1983), or into a pBluescript II vector (Stratagene). The fidelity of all PCR-generated clones was confirmed by sequencing. One of these constructs, pAC116-3, contained *IME1* (–2146 to –2017) in pBluescript II and was used as an intermediate for other constructions described below.

The *IME1-81* deletion allele was created by directed mutagenesis of pAC82 using the bottom strand oligonucleotide 5'-CATTTACTGCCATTTAGTTCGACATCCGCATTCTTG-3', which removed –2044 to –2025 and inserted one C (underlined) to create a *Sal*I restriction site. The region containing the mutation was cloned into the integrating vector pRS306 (Sikorski and Hieter 1989) to give pAC156. pAC156 was used to transform yeast in a two-step replacement that created *IME1-81* in the chromosome. This allele is marked with only the *Sal*I site, which was used to follow the mutation in genetic crosses.

RRE mutant isolation

RRE mutations were generated by mutagenic PCR amplification of pAC116-3 (Leung et al. 1989). Flanking *Kpn*I and *Sal*I sites derived from the polylinker were digested, and the products were ligated into the *Kpn*I and *Sal*I sites of pKB112, which eliminates UAS_{CYC1} sequences and gives inserts in a single orientation. Thus, a pool of plasmids with mutated inserts was collected. pAC119-10, the wild-type positive control, was made by ligating the wild-type fragment from pAC116-3 into the *Kpn*I and *Sal*I sites of pKB112.

The mutant plasmid pool was amplified in *Escherichia coli*

and transformed into yeast strain 1122. Transformants were replica plated onto selective galactose medium containing X-gal. Colony color was compared with that of colonies bearing the control plasmid pAC119-10. Plasmids from white colonies were retrieved in *E. coli* and retested in yeast strain 1122. The inserts from 38 clones were sequenced and found to contain one to six mutations including transitions, transversions, and deletions. Thirty contained at least one mutation in the RRE region. Two of the most frequently isolated transitions and one transversion were introduced singly by oligo-directed mutagenesis of pAC116-3. These mutant elements were then cloned into the *Kpn*I and *Sal*I sites of pKB112 to form pAC128-4, pAC119-1, and pAC119-2, which contain the RRE ↓ 1, RRE ↓ 2, and RRE ↓ 3 mutations, respectively. A transition not isolated previously, designated RRE ↑ 4, was introduced in pAC116-3 and similarly transferred to pKB112 to form pAC119-3. RRE ↓ 1, RRE ↓ 2, RRE ↓ 3, and RRE ↑ 4 were introduced separately by oligo-directed mutagenesis of pAC82 to their normal positions upstream of the plasmid *IME1* gene, thus forming pAC136, pAC118-1, pAC118-2, and pAC118-3, respectively.

Northern analysis

IME1 RNA expression from pAC82 and pAC85-26 was assayed in strains grown to early log phase in synthetic acetate–glycerol media lacking tryptophan and shifted to 2% potassium acetate plus auxotrophic amino acid supplements. RNA was prepared at 0, 2, and 4 hr after the shift. Electrophoresis, blotting, and hybridization conditions were as described previously (Smith and Mitchell 1989).

β-Galactosidase and sporulation assays

Assays of strains 1122 and 1124 bearing derivatives of the *cyc1-lacZ* reporter plasmids pKB112 or pLGΔ312SΔSS were performed on cells grown in synthetic galactose media lacking uracil to mid-log phase. *ime2-lacZ* expression, used to monitor *IME1* regulation (Smith et al. 1990), was assayed from cells grown to log phase in YEPac (veg) and shifted to 2% potassium acetate plus auxotrophic supplements (spo). Percent sporulation was measured by counting the number of asci per 200 cells after 2–3 days on sporulation medium. The reported values of all β-galactosidase and sporulation assays are the average of three determinations.

Protein extracts

Cells were grown in 500 ml of YEPac media to late log phase, harvested by centrifugation, washed once in H₂O and once in lysis buffer [200 mM Tris–H₂SO₄ at pH 7.9, 50 mM (NH₄)₂SO₄, 2 mM ZnSO₄, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol (vol/vol)]. All subsequent steps were performed on ice or at 4°C. Pellets were resuspended in 4 ml of lysis buffer with protease inhibitors (100 μM PMSF, 1 μg/ml of aprotinin, 0.5 μg/ml of leupeptin, 0.7 μg/ml of pepstatin) and 3 ml of acid-washed glass beads. Cells were broken by 20-sec pulses of vortexing for a total of 4–5 min. Debris was removed by centrifugation at 10,000g for 15 min, and the supernatant was then centrifuged at 106,000g for 1 hr to remove insoluble material. Polyethyleneimine (pH 7.6) was added to the supernatant to a final concentration of 0.08% (vol/vol). Precipitated nucleic acid was removed by centrifugation at 10,000g for 10 min. Solid (NH₄)₂SO₄ (0.35 grams/ml) of supernatant was added and dissolved slowly for 1 hr. Precipitated protein was collected by centrifugation at 10,000g for 20 min. Pellets were dissolved in storage buffer [50 mM Tris–HCl at pH 7.9, 50 mM KCl, 1 mM DTT, 25% (vol/vol)]

glycerol] with protease inhibitors and dialyzed against storage buffer with 100 μ M PMSF. Precipitated material was removed from the dialysate by centrifugation at 10,000g for 15 min. Extracts were adjusted to a concentration of 15 mg/ml of protein in storage buffer with protease inhibitors. Aliquots were frozen in a dry ice/ethanol bath, stored at -70°C , and thawed only once. The samples retained DNA-binding activity for up to 3 weeks.

Gel mobility retardation assays

The probe for mobility retardation assays was generated by filling in 5' overhangs of digested PCR products with [α - ^{32}P]dATP and Klenow enzyme. The DNA included 130 bp of *IME1* DNA (–2146 to –2017) and 10 bp of flanking polylinker sequence. Binding reactions contained 16 μ l of retardation buffer [50 mM Tris-HCl (pH 7.9) 50 mM KCl, 0.5 mM MgCl_2 , 250 μ g/ml of BSA, 1.5 mM DTT, 12% (vol/vol) glycerol], 1 μ g of poly[d(I-C)], 1 μ g of pBluescript II DNA, 0.25 ng (20,000 cpm) of radiolabeled probe, and 30 μ g of protein extract in a final volume of 20 μ l. For competition reactions retardation buffer volume was reduced to 15 μ l, and 250- or 500-fold excess of cold competitor in 1 μ l was added. Protein extract was added last in all reactions. Mixtures were incubated for 25 min at room temperature and loaded onto a prerun 5% polyacrylamide gel (29 : 1 acrylamide/bis) containing 2.5% glycerol (vol/vol), buffered with 0.5 $\times$ TBE. Samples were electrophoresed at 25 mA in 0.5 $\times$ TBE. Gels were dried and exposed to film overnight with an intensifying screen at -70°C .

Chemical nuclease footprinting

The source of the probe for the nuclease protection assay was pAC117-3, a site-directed mutant derivative of pAC116-3 that contains the RRE $\uparrow 4$ –2031 $\text{G} \rightarrow \text{A}$ mutation. A 210-bp fragment single end-labeled on the top strand was purified from digested pAC117-3 DNA. The probe included 130 bp of *IME1* DNA (–2146 to –2017 with RRE $\uparrow 4$) and 80 bp of polylinker sequence. In a scaled-up gel mobility retardation, 300 μ g of protein extract from strain 1269 [which carries *P_{GALI}*–*RME1* on the low-copy plasmid pAC34 (Covitz et al. 1991), as well as having a chromosomal copy] was added to 160 μ l of retardation buffer, 10 μ g poly[d(I-C)], 10 μ g of pBluescript II, and 400,000 cpm of probe DNA in a final volume of 200 μ l. Samples were incubated and electrophoresed as described above. The gel was wrapped in plastic and exposed to film without drying. Gel slices containing free probe DNA or RME1–DNA complex were cut out and placed in 5 ml of 10 mM Tris-HCl (pH 8.0). Next, 0.5 ml of 1,10-phenanthroline–copper ion reagent [5% 9 mM CuSO_4 , 5% 40 mM 1,10-phenanthroline in ethanol, 90% H_2O (vol/vol)] was added, followed by 0.5 ml of 0.5% mercaptopropionic acid (Kuwabara and Sigman 1987). The reaction proceeded for 30 min and was then quenched by adding 0.5 ml of 2,9-dimethyl-1,10-phenanthroline (5.8 mg/ml in ethanol). The gel slices were rinsed with water and placed in dialysis bags, each with 0.4 ml of TBE buffer. The DNA was electroeluted for 2 hr at 70 V, polarity was reversed for 2 min, and the liquid was recovered from the bags. After one phenol–chloroform–isoamyl alcohol extraction (25 : 24 : 1) 1 μ g carrier tRNA was added, and the samples were precipitated with ethanol. The pellets were rinsed with 80% ethanol and dried, and activity was determined by Cerenkov counting. Samples were dissolved in Maxam–Gilbert loading buffer (Maxam and Gilbert 1980), heated to 90°C for 5 min, and run on an 8% sequencing gel (31,000 cpm per lane). Maxam–Gilbert G and G + A reactions were run simultaneously as markers (Maxam and Gilbert 1980).

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Note added in proof

The updated *IME1* sequence has been submitted to the GenBank data library under accession number M37188.

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