Identification of factors involved in MSE-mediated repression of *SMK1*

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Abstract

Meiotic development in *Saccharomyces cerevisiae* is carried out by temporal regulation of distinct sets of genes. We have previously shown that a promoter element called the MSE, **m**iddle **s**porulation **e**lement, is responsible for the timing of expression of genes during the middle stages of sporulation. The role of many MSEs is to repress transcription of middle sporulation genes during vegetative growth and to activate transcription during meiotic growth. Previous work has shown that the Sum1, Hst1, and Rfm1 proteins are required for MSE-mediated repression. Interestingly, DNA microarray expression analysis shows that Sum1 is required for repression of a large number of middle sporulation genes while Hst1 and Rfm1 are required to repress only a subset of these genes. This result suggests that Sum1 may interact with other factors to repress genes not regulated by Hst1 and Rfm1. This study tries to identify factors that may interact with Sum1 to repress transcription during vegetative growth.

Introduction

Regulation of gene expression is very important during development and growth of an organism and is responsible for many of the critical cellular functions. One example of a developmental pathway that relies on the appropriate temporal expression of genes is the process of meiosis in yeast (Kupiec et al., 1997). This is the process in which a diploid cell undergoes a round of replication followed by two chromosomal divisions to produce four haploid nuclei that are packaged into spores. Genes required for these processes are expressed at specific stages in meiosis and are classified as early, middle, or late genes, depending on when they are expressed in the meiotic pathway (Mitchell, 1994). A question that arises is what determines the differential expression of these genes?

One factor that distinguishes expression of middle genes from early or late genes is the MSE, middle sporulation element (Bogengruber et al., 1998; Hepworth et al., 1995; Ozsarac et al., 1997). Many MSEs function as activator sites during middle sporulation and as repressor sites in vegetative growth (Pierce et al., 1998). Our

laboratory has shown that the Sum1, Hst1, and Rfm1 proteins are required for MSE-mediated repression of some middle sporulation genes (Xie et al., 1999; McCord et al., submitted). This finding provided the first indication of the normal function these proteins in the cell. *SUM1* had been previously identified as a dominant mutant, *SUM1-1*, that suppresses mutations in *sir2*, a gene required for transcriptional silencing of the silent mating type loci and telomeres (Chi and Shore, 1996; Klar et al., 1985; Laurenson and Rine, 1991). *HST1* had been identified as a sequence homolog to *SIR2*, a NAD+ dependent histone deacetylase; over-expression of this gene suppresses defects caused by a *sir2* mutation (Brachmann et al., 1995; Derbyshire et al., 1996). *RFM1* was previously an uncharacterized open reading frame (ORF) whose function was unknown. Sum1 has been shown to bind the *SMK1*-MSE *in vitro* and *in vivo* (Xie et al., 1999; Pierce, unpublished). Sum1 interacts with Rfm1, which in turn recruits Hst1 to the promoter. Hst1 is required for deacetylation of histones in the promoters of some middle genes and presumably sets up a repressed chromatin state (Rusche and Rine, 2001; McCord et al., submitted). Although the three proteins are required for MSE repression, a *sum1* null mutation has a much greater effect on repression than either *hst1* deletion or *rfm1* deletions. Expression analysis using DNA microarrays has shown that Sum1 regulates many of the middle sporulation genes, whereas Hst1 and Rfm1 are only required for a subset of the genes repressed by Sum1 (McCord et. al., submitted). These results raise the question of what other factors interact with Sum1 to repress this subset of genes? To answer this question, we performed a genetic screen to identify mutants that fail to repress *SMK1*, a middle sporulation gene that is repressed by Sum1 but is independent of Rfm1 and Hst1.

Materials and methods

SUM1 Mutagenesis and β-Galactosidase Assays Strains LNY385 and LNY433 (*MAT*α and MAT**a** versions of strain W303- *ade2-1 trp1-1 HIS3 can1-100 ura3-1 leu2-3, 112*) were transformed with pMDP83, a 2μ *URA3* plasmid that contains a *SMK1-lacZ* fusion (Pierce et al., 1998). These transformants were mutagenized by treatment with EMS for various lengths of time and the percent killing for each time point was determined by comparison to untreated cells (Xie et al., 1999). The time point that gave a 90% killing was used for further assays.

The mutagenized cells were diluted to obtain approximately 250 colonies per plate and were grown on SD-ura media for 2 days at 30°C. β-galactosidase filter lift assays were performed to determine if any transformants showed de-repression of the *SMK1-lacZ* fusion and expressed *lacZ* (Xie et al., 1999). Colonies that were blue on the filters were picked from the plate and struck out for single colonies. Three single isolates from each colony were picked and patched onto a fresh plate. Filter lift assays were repeated and clones that were blue were chosen for further assays.

Single point liquid β-galactosidase assays were performed using these clones to quantify levels of de-repression (Pierce et al., 1998). Mutants that showed greater then 2.0 units of b-galactosidase were mated with a wild-type strain of the opposite

mating type (LNY433 or LNY385) to determine if the mutation present was dominant or recessive. The same mutants were also mated with *sum1*Δ strain (JXY3 or JXY4) to determine whether the mutant contained a mutation in *SUM1* (Xie et al., 1999). Matings were performed by mixing cells of opposite mating types on YEPD plates followed by overnight growth at 30°C. Diploid cells were selected by replica plating to SD-ura-his plates and streaked for single colonies. Three single isolates were used to perform liquid β-galactosidase assays.

Results

Approximately 68,200 transformants were screened (**a**- 27,040 α- 41,180) at 90% killing. This translates into coverage of the yeast genome of approximately 11 times. The screen resulted in 332 blue transformants that were restreaked and retested by filter lift assay. This reduced the number of blue transformants to 81 (**a**- 55 α- 26). These mutants were renamed SWY1 through SWY81. To quantify the level of de-repression, single point liquid β-galactosidase assays were performed. These assays showed 22 of the 81 mutants had levels of β-galactosidase expression of 2.0 units or higher (Table 1). β-galactosidase assays were repeated on these 22 mutants using three single isolates from each mutant. Six of these mutants, SWY17, 20, 26, 31, 38, and 44, were only marginally above background and were not further characterized.

The 22 mutants were crossed with the wild-type LNY385 or LNY433 parent strain to create a heterozygous diploid . Three isolates of each diploid were picked and the level of de-repression of the reporter was quantified by liquid β-galactosidase assays. All of the diploid strains showed full repression of the *SMK1-lacZ* reporter plasmid indicating that the mutants are recessive to wild type (Table 1).

Twelve out of the 22 strains showed strong de-repression of the reporter promoter. These 12 strains were then mated with JXY3 and JXY4 to determine whether or not the mutations could be complemented by a *sum1* deletion strain. The inability of the resulting diploid to complement the de-repression phenotype would indicate the mutation present in the strain isolated from the screen is within *SUM1*. Diploid strains that repressed the *SMK1-lacZ* reporter would indicate a mutation to be present in a gene other then *SUM1*. b-galactosidase assays showed that only one (SWY57) out of the 12 mutants was complemented by the *SUM1* deletion strain (Table 1). These results suggest that the other 11 SWY mutants have mutations in *SUM1*.

SWY57 originally gave 11.1 units in the β-galactosidase assay when it was first isolated. However, repeating this b-galactosidase assay with three isolates of SWY57 gave 2.4 units. This change was troubling and suggested that there might have been a mutation in the reporter plasmid rather than in the genome. To test this possibility we introduced a fresh copy of the *SMK1-lacZ* reporter plasmid into SWY57. This was accomplished by first selecting for cells that have lost the reporter plasmid by growing SWY57/pMDP83 in non-selective YEPD liquid media followed by plating on 5'-FOA plates (Boeke et al., 1987). A colony that grew on this plate was then re-transformed with the reporter followed by β-galactosidase assay. Three isolates from

this transformation gave an average of 0.6 units, indicating the promoter was strongly repressed. This result indicates the SWY57 mutant isolated from the screen may have contained a mutation in the reporter plasmid that prevented repression.

Discussion

MSEs have an important role in regulating many middle sporulation genes in yeast. During vegetative growth many MSEs function as Sum1-dependent repressor sites to prevent expression (Pierce et al., 1998). Sum1 is an MSE DNA-binding protein that has been shown to interact with both Rfm1 and Hst1 cofactors. Given the potential histone deacetylase activity of Hst1, this complex could be used to modify chromatin structure and repress transcription at MSE containing promoters. However, we have shown that a subset of Sum1 regulated genes do not require Rfm1 and Hst1 for repression (Xie et al., 1999); Pierce et al. submitted; McCord et. al., submitted). This result suggested that Sum1 may recruit other cofactors to these promoters to repress transcription. We have tried to identify these cofactors using a screen designed to isolate mutants that fail to repress transcription of *SMK1*, a gene that is regulated by Sum1 but independent of Rfm1 and Hst1. Although the number of transformants screened covered the genome 11 times we obtained mutations only in *SUM1*. There are several explanations for our results.

One possibility is that although other proteins may be working with Sum1 these proteins may have a redundant function with other proteins in the cell. Therefore to observe de-repression of the *SMK1-LacZ* reporter, both genes that code for these redundant proteins would have to be mutated. The mutatational frequency in our screen generated roughly six mutations per cell. The chances of isolating a strain with mutation in both of these redundant genes would therefore be extremely rare.

A second possibility is that the protein working with Sum1 may have an essential function in the cell. Null mutations in this proteins would be lethal, making it impossible to isolate these mutants. In support of this model is the observation that *SUM1-1*interacts with ORC at *HMR* to suppress the affects of a *sir2* mutation (Rusche and Rine, 2001; Sutton et al., 2001). ORC, the origin replicating complex, is involved in many essential functions in the cell including associating with the origins of DNA replication. Mutations in many of the proteins that form the ORC have a lethal phenotype. If Sum1 requires ORC to repress pMDP83, then it is unlikely that we would have isolated mutations in these genes in our screen. One way to test this model is to use conditional temperature sensitive mutations in ORC proteins to determine if the *SMK1-lacZ* reporter is de-repressed under non-permissive conditions. We are currently conducting these experiments to test this hypothesis.

A third explanation for why only *sum1* mutants were isolated in our screen is the possibility that Sum1 is acting alone by steric interference at the promoter region. For example, if Sum1 binding to the MSE blocks access to the binding sites for proteins that are required for transcriptional activation then the promoter will be repressed. Therefore, simply removing *SUM1* would allow for binding by the activator protein

and expression of the reporter. In support of this model, the MSE in the *SMK1* promoter is only 40 base pairs from the UAS activator site and 10 base pairs from a predicted TATA element. Binding by Sum1 to the MSE may therefore inhibit recognition of these sites by these other factors.

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Table 1. Expression of the SMK1-lacZ reporter in mutant strains

SWY Strain	**Single PointHaploid Strain**(Units of β-galactosidase)	**Three PointHaploid Strain**(Units of β-galactosidase)	**Wild-Type Mated With Haploid**(Units of β-galactosidase)	***sum1* Deletion Mated With Haploid**(Units of β-galactosidase)
WT	0.8	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
17	5.0	1.4 ± 0.1	0.7 ± 0.2	
19	17.8	6.7 ± 0.6	0.9 ± 0.4	
20	3.1	0.6 ± 0.1	0.8 ± 0.2	
26	6.2	1.1 ± 0.1	0.7 ± 0.1	
31	4.3	1.5 ± 0.1	0.8 ± 0.2	
32	57.7	38.7 ± 1.7	0.8 ± 0.1	21.6 ± 3.7
33	5.1	5.7 ± 1.5	0.5 ± 0.1	
37	9.0	7.8 ± 0.4	0.5 ± 0.1	
38	4.5	1.4 ± 0.4	0.5 ± 0.2	
44	6.7	1.6 ± 0.2	0.8 ± 0.4	
45	140.4	113.1 ± 28.1	1.0 ± 0.3	22.8 ± 5.3
47	45.0	86.4 ± 26.6	1.4 ± 0.4	21.9 ± 9.2
48	46.0	63 ± 6.1	1.1 ± 0.3	18.3 ± 3.9
51	69.1	87.8 ± 5.0	1.3 ± 0.2	20.8 ± 5.1
54	35.6	73.2 ± 45.5	1.0 ± 0.4	19.9 ± 14.0
56	19.7	13.1 ± 0.9	0.9 ± 0.4	12.5 ± 1.9
57	11.1	2.4 ± 0.3	0.7 ± 0.2	0.5 ± 0.0
62	3.6	5.2 ± 0.2	0.4 ± 0.1	
63	39.6	47.0 ± 2.6	0.6 ± 0.0	32.7 ±10.7
77	36.3	29.4 ± 6.7	0.8 ± 0.1	18.4 ± 5.1
78	78.4	75.9 ±15.3	0.7 ± 0.1	24.8 ± 9.8
81	134.9	139.8 ± 45.6	1.5 ± 1.4	25.2 ± 7.6