SPE3, Which Encodes Spermidine Synthase, Is Required for Full Repression Through NRE^{DIT} in *Saccharomyces cerevisiae*

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ABSTRACT

We previously identified a transcriptional regulatory element, which we call NRE^{DIT}, that is required for repression of the sporulation-specific genes, *DIT1* and *DIT2*, during vegetative growth of *Saccharomyces cerevisiae*. Repression through this element is dependent on the Ssn6-Tup1 corepressor. In this study, we show that *SIN4* contributes to NRE^{DIT}-mediated repression, suggesting that changes in chromatin structure are, at least in part, responsible for regulation of *DIT* gene expression. In a screen for additional genes that function in repression of *DIT* (*FRD* genes), we recovered alleles of *TUP1*, *SSN6*, *SIN4*, and *ROX3* and identified mutations comprising eight complementation groups of *FRD* genes. Four of these *FRD* genes appeared to act specifically in NRE^{DIT}-mediated repression, and four appeared to be general regulators of gene expression. We cloned the gene complementing the *frd3-1* phenotype and found that it was identical to *SPE3*, which encodes spermidine synthase. Mutant *spe3* cells not only failed to support complete repression through NRE^{DIT} but also had modest defects in repression of some other genes. Addition of spermidine to the medium partially restored repression to *spe3* cells, indicating that spermidine may play a role *in vivo* as a modulator of gene expression. We suggest various mechanisms by which spermidine could act to repress gene expression.

SPORULATION of the yeast *Saccharomyces cerevisiae* is a process of cellular differentiation that begins when *MAT***a**/*MAT*α diploid cells are starved in the presence of a nonfermentable carbon source. As a cell progresses through the events of meiosis and spore wall formation, an ordered series of genetic and morphological changes generates a tetrad of dormant haploid spores that are resistant to environmental insults. A single round of DNA replication is followed by a lengthy prophase during which homologous chromosomes pair and undergo high levels of meiotic recombination. The two meiotic divisions, leading to segregation of homologous chromosomes and then sister chromatids, occur within the nucleus. Prospore membranes begin to form at the spindle pole bodies and expand to engulf each daughter nucleus, as well as some cytoplasm. Deposition of spore wall material then generates a multilayered spore wall, giving rise to four mature spores within the ascal sac (reviewed in Kupiec *et al.* 1997). The process of spore formation is associated with expression of ≥4 temporally distinct classes of sporulation-specific genes, referred to as early, middle, mid-late, and late on the basis of their time of expression (reviewed in Mitchell 1994; Kupiec *et al.* 1997). Sporulation in *S. cerevisiae*, therefore, provides a useful model for studying the temporal control of gene expression during development.

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The mid-late sporulation-specific genes are first activated around the time that the meiotic divisions are being completed and synthesis of the spore membrane has begun. The divergently transcribed genes, *DIT1* and *DIT2*, are the only mid-late sporulation-specific genes thus far identified. These genes encode enzymes that are required for biosynthesis of the dityrosine precursor that is incorporated into the outermost layer of the spore wall (Briza *et al.* 1990, 1994). *DIT1* and *DIT2* are repressed during vegetative growth via a common negative regulatory element, referred to as NRE^{DIT} (Friesen *et al.* 1997). Repression of the *DIT1* and *DIT2* genes during vegetative growth depends on the Ssn6-Tup1 corepressor acting through NRE^{DIT} (Friesen *et al.* 1997). We presume that a putative NRE^{DIT}-binding protein recruits the corepressor to the regulatory region of the *DIT* genes; it is possible, however, that the effect of Ssn6-Tup1 is indirect. NRE^{DIT} itself is bipartite in nature. One region has similarity to a middle sporulation element (MSE), an element that suffices for activation of middle sporulation-specific genes (Hepworth *et al.* 1995; Ozsarac *et al.* 1997) This MSE-like element from the *DIT* promoter is required for high levels of expression during sporulation in the context of the entire *DIT* promoter, but has no activity on its own. The adjacent region is essential for repression (Friesen *et al.* 1997). Regulation of expression of the *DIT* genes is complex; a high level of sporulation-specific gene expression requires at least two downstream elements in addition to NRE^{DIT} (Friesen *et al.* 1997).

Repression mediated in yeast by the Ssn6-Tup1 core-

pressor has been studied extensively. Ssn6 (Cyc8) and Tup1 are involved directly in the repression of genes regulated by glucose and by cell type and have been implicated in the direct repression of genes regulated by oxygen and by DNA damage, as well as genes involved in flocculation (Mukai *et al.* 1991; Keleher *et al.* 1992; Zhou and Elledge 1992; Zitomer and Lowry 1992; Elledge *et al.* 1993; DeRisi *et al.* 1997). Genetic and biochemical evidence indicates that Ssn6 and Tup1, neither of which binds to DNA, associate in a complex that is recruited to the promoters of coordinately regulated genes by pathway-specific DNA-binding proteins (Williams *et al.* 1991; Keleher *et al.* 1992; Tzamarias and Struhl 1994; Smith *et al.* 1995; Treitel and Carlson 1995; Tzamarias and Struhl 1995; Varanasi *et al.* 1996; Redd *et al.* 1997; reviewed in Roth 1995; Struhl 1995). Two models have been proposed for the mechanism of Ssn6-Tup1-mediated repression. In one model, Ssn6-Tup1 is thought to repress transcription by directing alterations in chromatin structure. In support of this model, Ssn6-Tup1-dependent repression is associated with positioned nucleosomes in the promoters of *SUC2* (Matallana *et al.* 1992) and *STE6* (Cooper *et al.* 1994). In addition, Tup1 has been shown to interact with histones H3 and H4 *in vitro* (Edmondson *et al.* 1996). Ssn6-Tup1 is also thought to mediate repression through effects on the general transcription machinery. In support of this model, partial Ssn6-Tup1-dependent repression of transcription can be recreated *in vitro* in reactions that contain naked DNA templates (Herschbach *et al.* 1994; Redd *et al.* 1997). It is likely that Ssn6-Tup1 mediates repression in several ways, with direct and indirect mechanisms making different contributions at different promoters (Huang *et al.* 1997).

In this article, we report the identification and preliminary characterization of genes that are required for complete repression through NRE^{DIT}, the Ssn6-Tup1-dependent operator controlling mid-late sporulation-specific gene expression. One of these genes is identical to *SPE3*, which encodes spermidine synthase. We found that cells that could not synthesize spermidine not only failed to support complete repression through NRE^{DIT} but also had modest defects in repression of other genes. Because addition of spermidine to the medium partially restored repression to *spe3* cells, we suggest that spermidine may have a role *in vivo* as a modulator of gene expression.

MATERIALS AND METHODS

Media, growth conditions and genetic methods: Liquid and solid media have been described (Hepworth *et al.* 1995). Sporulation medium consisted of 1% potassium acetate supplemented with the required auxotrophic supplements. Synthetic medium (SD), also referred to as minimal medium, contained 0.7% yeast nitrogen base without amino acids, auxotrophic supplements [40 μg of adenine sulfate/ml, 20 μg of arginine (HCl)/ml, 20 μg of histidine/ml, 60 μg of leucine/ml, 30 μg of lysine (mono HCl)/ml, 20 μg of methionine/ml, 50 μg of phenylalanine/ml, 200 μg of threonine/ml, 40 μg of tryptophan/ml, 30 μg of tyrosine/ml, and 20 μg of uracil/ml], and 2% glucose. For sporulation, yeast strains were grown at 30° in minimal medium (SD) to midlog phase, and the cells were then harvested, washed, and transferred to sporulation medium at a density of $\sim 2 \times 10^7$ cells per ml. The time of transfer of cells to sporulation medium is referred to as 0 hr. Standard genetic methods were employed for mating, sporulation, and tetrad analysis (Sherman 1991). Yeast cells were transformed by the lithium acetate method (Gietz *et al.* 1992).

Strains: *S. cerevisiae* strains used in this study are listed in Table 1. EG123, EG123tup1, and EG123ssn6 were provided by A. Johnson and have been described (Schultz *et al.* 1990; Keleher *et al.* 1992). All other strains were derived from W303-1A and W303-1B. The **a**/α diploid strain obtained by mating W303-1A and W303-1B is referred to as LP112. DY1702 is a derivative of W303-1A in which the *SIN4* gene has been replaced with the *sin4*Δ*::TRP1* allele (Jiang and Stillman 1992) and was provided by D. Stillman. The mutants whose isolation is described in this article are named after the defective allele; *e.g.*, the mutant containing the *frd3-1* allele is named Yfrd3-1. Homozygous mutant diploid strains, referred to as YYfrd, were obtained as follows: First, the Yfrd strains were mated with strain W303-1A containing pLG+NRE76, and diploids were selected on SD-Trp-Ura. These heterozygous diploids were sporulated, and Ura$^+$ Trp$^-$ colonies derived from *MAT***a** spores that contained the *frd* mutant allele were identified by their defect in repression of the reporter gene. The *MAT***a** *frd* mutants were mated back to the original *MAT*α *frd* mutant strains, and homozygous diploids were selected on SD-Trp-Ura.

Yspe3::HIS3α was constructed in two steps. First, the wild-type diploid strain LP112 was transformed with a 10.3-kb *Xba*I-*Xba*I fragment that had been isolated from pG23Tn42 and that contained an *spe3::HIS3* allele. Replacement in a His$^+$ transformant of one copy of *SPE3* by the *spe3::HIS3* allele was confirmed by Southern blot analysis of DNA digested with *Bgl*II. The resultant strain was called LP112spe3::HIS3. The *spe3::HIS3* allele, which had a Tn1000::HIS3 element (Morgan *et al.* 1996) inserted 187 nt downstream of the ATG of the *SPE3* gene, did not complement the *frd3-1* mutation. Second, Yspe3::HIS3α was obtained by sporulation of cells of LP112spe3::HIS3 that had been transformed with pLG+ NRE76. Progeny derived from a haploid *MAT*α spore that failed to fully repress the *CYC1-NRE*^{DIT}*-lacZ* reporter gene were grown in the presence of 5-fluoroorotic acid (5-FOA) (Boeke *et al.* 1984) to select segregants that had lost pLG+NRE76, generating the strain Yspe3::HIS3α.

WA-ROX3-LEU2 was constructed by transforming W303-1A with pRS305-ROX3 (see below) that had been digested with *Bgl*II. Integration at the *ROX3* locus was confirmed by Southern blot analysis of DNA from Leu$^+$ transformants. Yfrd3-1 and Yspe3::HIS3α strains containing an integrated *CYC1-lacZ* reporter gene or an integrated *CYC1-NRE*^{DIT} *lacZ* reporter gene were constructed by transformation with YIpLG312 and YIpLG+NRE76 (see below) that had been digested with *Stu*I to target integration to the *URA3* locus (Keleher *et al.* 1992). Strains that had a single copy of the reporter gene were identified by Southern blot analysis.

The *Escherichia coli* strain DH5α was used for propagating plasmids. Strain MC1066 [*pyrF74::Tn5*(*Km*^{r}) *leuB trp*] was used to select for plasmids containing the yeast *LEU2* marker (Casadaban *et al.* 1983).

Plasmids: Nonstandard plasmids used in this study are listed in Table 2. Throughout this work, we refer to pLGΔ312(Bgl) (provided by A. Mitchell), which is a derivative of pLGΔ312

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype	Source
Haploids		
W303-1BT	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100*	
W303-1A	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100*	
Yfrd1-1	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd1-1*	This work
Yfrd2-1	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd2-1*	This work
Yfrd3-1	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd3-1*	This work
Yfrd4-1	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd4-1*	This work
Yfrd5-1	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd5-1*	This work
DY1702	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 sin4*Δ*::TRP1*	D. Stillman
EG123	*MAT***a** *trp1 leu2 ura3 his4*	A. Johnson
EG123tup1	*MAT***a** *trp1 leu2 ura3 his4 tup1*Δ*::LEU2*	A. Johnson
EG123ssn6	*MAT***a** *trp1 leu2 ura3 his4 ssn6*Δ*9*	A. Johnson
WA-ROX3-LEU2	W303-1A with pRS305-ROX3 inserted at *ROX3* locus	This work
Yspe3::HIS3α	*MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 spe3::HIS3*	This work
Diploids		
LP112	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100*	This work
YYfrd1-1	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd1-1* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd1-1*	This work
YYfrd2-1	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd2-1* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd2-1*	This work
YYfrd3-1	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd3-1* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd3-1*	This work
YYfrd4-1	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd4-1* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd4-1*	This work
YYfrd5-1	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd5-1* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 ura3-1 can1-100 frd5-1*	This work
LP112spe3::HIS3	*MAT***a** *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 spe3::HIS3* *MAT*α *ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 SPE3*	This work

(Guarente and Mason 1983), as pLG312 and to the reporter gene on this plasmid as *CYC1-lacZ*. pLG312 contains a unique *Bgl*II site, flanked by *Sal*I and *Xho*I sites, at nucleotide −178, which is located between the *CYC1* UASs and the TATA box of the *CYC1-lacZ* fusion gene; a unique *Sma*I site is located at nucleotide −312, upstream of the upstream activating sequences (UASs). The plasmid pLG+NRE76, which contains the reporter gene referred to as *CYC1-NRE*^{DIT}*-lacZ*, has a 76-bp fragment containing NRE^{DIT} inserted into the *Bgl*II site of pLG312 (Friesen *et al.* 1997).

Plasmid pLG+NRE30 was constructed by annealing the oligonucleotides 5′-GATCCGGGTTCTCTTGCCAAGAAAAAAT AAAAAGG-3′ and 5′-GATCCCTTTTTATTTTTTCTTGGCAA GAGAACCCG-3′ and cloning the double-stranded fragment into the *Bgl*II site of pLG312 (Guarente and Mason 1983).

pRS305-ROX3 was constructed by subcloning an ∼2.7-kb *Hin*dIII-*Hin*dIII fragment containing *ROX3* from YCp(33)-ROX3H (a gift from Richard Zitomer) into the *Hin*dIII site of pRS305 (Sikorski and Hieter 1989). pSPE3 · LEU2 was constructed by cloning the 2.9-kb *Bgl*II-*Bgl*II fragment of pG23, which contains the *SPE3* gene, into the *Bam*HI site of pRS315 (Sikorski and Hieter 1989). YIpLG312 and YIpLG+NRE76 were constructed from pLG312 and pLG+NRE76 by digestion with *Hin*dIII and religation, resulting in the deletion of an ∼2-kb fragment containing 2-μm sequences.

Isolation of *frd* mutants: Strain W303-1BT (*MAT*α) containing pLG+NRE76 was mutagenized to 76% survival with ethyl methanesulfonate (EMS) as described (Lawrence 1991). Mutagenized cells were plated on SD-Ura at a density of ∼800 colony-forming units per plate and incubated at 30° for 2–3 days, at which time the colonies were overlaid with X-Gal-containing agar (see β-galactosidase assays below). After an additional ∼18 hr incubation, cells recovered from colonies that appeared blue were patched in duplicate onto SD-Ura. After growth at 30° for 1–3 days, one set of colonies was retested by the X-Gal overlay assay for derepression of the *CYC1-NRE*^{DIT}*-lacZ* reporter gene. Of ∼60,000 colonies tested, 16 colonies that appeared blue on retesting were picked for further study.

Genetic analysis: Mutants were placed into complementation groups using standard techniques (Sherman 1991). Allelism with *SSN6* and *TUP1* was assessed by mating mutants containing pLG+NRE76 with the isogenic strains EG123, EG123ssn6, and EG123tup1 and testing the resulting diploids for repression of the *CYC1-NRE*^{DIT}*-lacZ* reporter gene by the overlay assay. Allelism with *SIN4* was assessed in the same way after mating mutants with W303-1A and the isogenic *sin4*Δ strain, DY1702. Allelism with *ROX3* was assessed by mating Yfrd13-1 containing pLG+NRE76 with WA-ROX3-LEU2 and analyzing tetrads derived from the resulting diploid strain.

To monitor the relative level of expression of various *lacZ* reporter genes in the mutant strains, cells that had lost pLG+NRE76 were first selected on medium that contained 5-FOA (Boeke *et al.* 1984). Ura$^-$ derivatives of each mutant were then transformed with pLG312, pLG+NRE76, pLGΔSS, pLG+α2op, and p(−537)DIT1-lacZ. Transformants were patched on SD-Ura plates and incubated at 30°. Patches that had been overlaid with X-Gal-containing agar were examined for relative blueness after 18 hr incubation at 30°.

TABLE 2

Plasmids

Name	Description	Source
pLG312	*CYC1-lacZ* reporter, *URA3*, 2-μm origin	Guarente and Mason (1983)
pLGΔSS	*CYC1-lacZ* reporter with *CYC1* UASs deleted	Guarente and Hoar (1984)
pLG+NRE76	pLG312 with a 76-bp fragment containing NRE^{DIT} (nt −537 to −462 of *DIT1*) inserted between the UASs and TATA of the *CYC1* promoter	Friesen *et al.* (1997)
pAJ3 (pLG+α2op)[a]	pLG312 with the α2-Mcm1 site inserted between the UASs and TATA of the *CYC1* promoter	Keleher *et al.* (1992)
p(−537)DIT1-lacZ	*DIT1-lacZ* fusion containing sequences from nt −537 to +53 of *DIT1* fused to *lacZ*, *URA3*, 2-μm origin	Friesen *et al.* (1997)
pRS305-ROX3	pRS305 containing a ∼2.7-kb *Hin*dIII-*Hin*dIII fragment containing *ROX3*	This work
pLG+2×NRE76/S	pLG312 with two copies of a 76-bp fragment containing NRE^{DIT} inserted upstream of the *CYC1* UASs	Friesen *et al.* (1997)
pLG+NRE30	pLG312 with a 30-bp fragment (nt −493 to −464) inserted between the UASs and TATA of the *CYC1* promoter	This work
pG23	Plasmid containing *SPE3* isolated from a p366-based (*CEN4 ARS1*) yeast genomic library (Rose and Broach 1991)	This work
pG51	Plasmid containing *SPE3* isolated from a p366-based yeast genomic library	This work
pG23Tn42	pG23 with transposon insertion at nt +187 (where +1 is the ATG) of *SPE3* gene	This work
pG23Tn44	pG23 with transposon insertion at nt +370 of *SPE3* gene	This work
pG23Tn40	pG23 with transposon insertion at nt +692 of *SPE3* gene	This work
pSPE3 · LEU2	2.9-kb *Bgl*II-*Bgl*II fragment from pG23 containing the *SPE3* gene subcloned into pRS315	This work
YIpLG312	pLG312 with 2-μm sequences deleted	This work
YIpLG+NRE76	pLG+NRE76 with 2-μm sequenced deleted	This work

[a] Parentheses indicate that the plasmid is referred to by its pLG name in this study.

β-Galactosidase assays: β-Galactosidase activity was measured in extracts of cells as described (Hepworth *et al.* 1995). Cells were grown to late log phase in SD-Ura, and then diluted and grown for an additional three to four generations in the same medium before being harvested. The activities reported are averages obtained from three to six cultures. We repeated each experiment one to three times and consistently found that the relative levels of β-galactosidase activity were similar from one experiment to the next. β-Galactosidase activity is given in nanomoles of *o*-nitrophenyl-β-d-galactopyranoside (ONPG) cleaved per min per mg protein at 28°.

The X-Gal overlay assay has been described previously (Barral *et al.* 1995). We used 0.2 mg X-Gal per ml in top agar in the screen for mutants and 0.4 mg X-Gal per ml in top agar for all subsequent experiments. We found that viable cells could be recovered from 80 to 95% of the overlaid colonies after 18 hr incubation; after 40 hr incubation, we could recover viable cells from less than 30% of the colonies (data not shown).

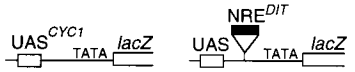
Cloning of *FRD3*: Strain Yfrd3-1 containing pLG+NRE76 was transformed with a p366-based (*CEN4 ARS1*) yeast genomic library (ATCC, a gift of N. Macpherson and B. Andrews; described in Rose and Broach 1991). Twenty-four thousand transformants were plated on SD-Leu-Ura medium at a density of ∼200 transformants per plate, and the plates were incubated for 3–4 days at 30°. After the colonies had been overlaid with X-Gal-containing agar (see β-galactosidase assays above) and incubated for an additional 18 hr at 30°, cells were recovered from colonies that remained white. On retesting, two transformants were identified that repressed the *CYC1-*NRE^{DIT}*-lacZ* reporter gene and appeared to have no growth defect. Complementation by the p366-based library plasmids was confirmed by passaging them through MC1066, a *leuB* strain of *E. coli* (Casadaban *et al.* 1983) and reintroducing the plasmids into Yfrd3-1.

DNA sequencing: The junctions between vector and insert in pG23 and pG51, plasmids which complemented the *frd3-1* mutation, were determined by dideoxy sequence analysis of double-stranded DNA (Sanger *et al.* 1977). The primers used, pBR-355T (5′-GGCGACCACACCCGTCCT-3′) and pBR-394B (5′-GCGTCCGGCGTAGAGGAT-3′), flank the *Bam*HI site of pBR322 and of its derivative, p366. The sequence was compared to the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/) to identify the chromosomal region that was present in the complementing plasmids.

Transposon mutagenesis: Tn1000 (γδ) transposon mutagenesis has been described (Morgan *et al.* 1996; Sedgwick and Morgan 1994). Plasmid DNA was isolated from 24 colonies and transformed individually into Yfrd3-1 that contained pLG+NRE76. The site of insertion of the transposon was identified by sequencing from primers within the transposon as described (Morgan *et al.* 1996; Sedgwick and Morgan 1994) and comparing the sequence with that in the Saccharomyces Genome Database.

RESULTS

In a previous study, we found that multiple regulatory elements within the promoter region of the *DIT1* gene of *S. cerevisiae* contribute to its sporulation-specific expression (Friesen *et al.* 1997). One of these elements, termed NRE^{DIT}, acts as an operator to prevent expression of the *DIT1* gene in vegetatively growing cells. This ele-



Genotype	pLG312	pLG+NRE76	Repression
wild type	1600	6.5	250 X
*sin4*Δ	2300	110	21 X

Figure 1.—NRE^{DIT}-mediated repression of the *CYC1-lacZ* reporter gene is reduced in a *sin4*Δ strain. Cells of strains W303-1A (wild-type) and DY1702 (*sin4*Δ) bearing either pLG312 or pLG+NRE76 were grown in SD-Ura, harvested, and assayed for β-galactosidase activity. Units of β-galactosidase are the averages of assays performed on at least three independent cultures. Fold repression refers to the β-galactosidase activity obtained in the strain containing pLG312 divided by the β-galactosidase activity obtained in the strain containing pLG+NRE76.

ment is also effective in preventing expression of a *CYC1-lacZ* reporter gene, with this effect being independent of the site of insertion of NRE^{DIT} and of its orientation (Friesen *et al.* 1997). In the current study, we have carried out a genetic screen to identify mutants that are defective in mediating NRE^{DIT}-dependent repression in vegetative cells. We anticipated that this screen would identify genes that are required specifically for repression through NRE^{DIT} and genes that serve a general role in repression of gene expression. In our experiments, we compared the efficiency of NRE^{DIT}-mediated repression in various strains by assessing the expression of two reporter genes, which we refer to as the *CYC1-lacZ* gene and the *CYC1-NRE^{DIT}-lacZ* gene. The *CYC1-lacZ* reporter gene contains the *CYC1* UASs and TATA box and is present in pLG312; the *CYC1-NRE^{DIT}-lacZ* reporter gene contains a 76-bp fragment containing NRE^{DIT} inserted between the *CYC1* UASs and TATA box of the *CYC1-lacZ* gene and is present in pLG+NRE76.

***SIN4* contributes to NRE^{DIT}-mediated repression:** Because we had previously found that repression through NRE^{DIT} requires the Ssn6-Tup1 corepressor (Friesen *et al.* 1997) and because *SIN4*, which modulates expression of various genes (Jiang and Stillman 1992; Chen *et al.* 1993; Covitz *et al.* 1994), is required for full repression of several Ssn6-Tup1-regulated genes (Chen *et al.* 1993; Wahi and Johnson 1995; Song *et al.* 1996), we tested whether *SIN4* contributed to NRE^{DIT}-mediated repression. Comparison of β-galactosidase expression from plasmid-borne *CYC1-lacZ* and *CYC1-NRE^{DIT}-lacZ* reporter genes introduced into isogenic wild-type (W303-1A) and *sin4*Δ (DY1702) cells indicated that NRE^{DIT}-mediated repression was reduced 12-fold in *sin4* cells (Figure 1); this effect is similar to the ninefold reduction in repression from the α2-Mcm1 operator in *sin4* cells (Wahi and Johnson 1995). We conclude that *SIN4* is required to achieve maximal repression through NRE^{DIT}.

Isolation of mutants with defects in repression through NRE^{DIT}: To identify additional genes that might be involved in mediating NRE^{DIT}-dependent repression, we monitored expression of a plasmid-borne *CYC1-NRE^{DIT}-lacZ* reporter gene in cells that had been exposed to the mutagen EMS. By using an overlay assay to detect β-galactosidase activity in colonies of cells (Barral *et al.* 1995), we identified 16 strains from ~60,000 survivors of mutagenesis that expressed the reporter gene. Complementation analysis placed 15 of these mutants into 12 complementation groups, named *frd1* through *frd13*, referring to the fact that the wild-type gene functions in repression of *DIT* (*FRD*) genes (Table 3; data not shown). Each mutant strain was named according to its defective allele; for example, Yfrd1-1 is a haploid mutant strain that contains the *frd1-1* allele, and YYfrd1-1 is an **a**/α diploid homozygous for the *frd1-1* allele. One mutant strain, which was completely defective in mating, and therefore could not be placed in a complementation group, was not characterized.

Because NRE^{DIT}-mediated repression requires the corepressor Ssn6-Tup1 (Friesen *et al.* 1997) and Sin4 (see above), we determined whether any of our strains contained mutant alleles of *SSN6*, *TUP1*, or *SIN4*. By testing diploid *frdX*/*ssn6*Δ, *frdX*/*tup1*Δ, and *frdX*/*sin4*Δ strains (Jiang and Stillman 1992; Keleher *et al.* 1992) for expression of the *CYC1-NRE^{DIT}-lacZ* reporter gene with an X-Gal overlay assay, we found that three strains, Yfrd7-1, Yfrd7-2, and Yfrd7-3, contained mutant alleles of *SSN6*, one strain, Yfrd8-1, contained a mutant allele of *TUP1*, and two strains, Yfrd6-1 and Yfrd6-2, contained mutant alleles of *SIN4* (Table 3). The identification of mutant alleles of *SSN6*, *TUP1*, and *SIN4* indicated that this screen could indeed lead to the isolation of genes required for repression through NRE^{DIT}.

Expression of a *CYC1-lacZ* reporter gene lacking a UAS is elevated in class I mutants: As the first step in the preliminary characterization of the Yfrd strains, we determined whether reduced repression through NRE^{DIT} could be accounted for by a defect in repression of basal transcription. We assessed basal transcription by monitoring expression of a plasmid-borne *CYC1-lacZ* reporter gene that lacks a UAS. This reporter gene was not expressed in the wild-type strain as monitored by an X-Gal overlay assay, but was expressed in Yfrd11-1, Yfrd12-1, and Yfrd13-1 and, as expected, in Yfrd6-1 and Yfrd6-2, strains that had mutant alleles of *SIN4* (Table 3; pLGΔSS column). We refer to these strains as class I mutants.

We tested several genes that are known to have a role in repressing basal transcription for identity with class I genes. We found that a plasmid-borne version of *ROX3*/*SSN7* complemented the *frd13-1* allele. *ROX3* is required for repression of other Ssn6-Tup1-regulated genes [*CYC7* (Rosenblum-Vos *et al.* 1991); *SUC2* (Song *et al.* 1996); and *MFA2* (Wahi and Johnson 1995; Carlson 1997)] and has recently been shown to encode a component of the mediator complex of RNA polymerase II

TABLE 3

Relative expression of various reporter genes in wild-type and mutant strains

Strain	Mutation in[b]	Reporter gene[a]				
		pLG312	pLG+NRE76	pLGΔSS	pLG+α2op	pDIT1-lacZ
Wild type		b	w	w	w	w
Class I mutants[c]						
NRE^{DIT-}α2-$Mcm1^{-}basal^{-}$						
Yfrd6-1	*SIN4*	b	b	pb	b	b
Yfrd6-2	*SIN4*	b	pb	pb	pb	b
Yfrd11-1		b	pb	vpb	pb	pb
Yfrd12-1		b	b	vpb	b	b
Yfrd13-1	*ROX3*	b	b	b	b	b
Class II mutants						
NRE^{DIT-}α2-$Mcm1^{-}basal^{+}$						
Yfrd7-1	*SSN6*	b	b	w	b	b
Yfrd7-2	*SSN6*	b	b	w	b	b
Yfrd7-3	*SSN6*	b	b	w	b	b
Yfrd8-1	*TUP1*	b	b	w	b	w
Yfrd10-1		b	b	w	vpb	w
Class III mutants						
NRE^{DIT-}α2-$Mcm1^{+}basal^{+}$						
Yfrd1-1		b	b	w	w	w
Yfrd2-1		b	b	w	w	w
Yfrd3-1		b	b	w	w	w
Yfrd4-1		b	b	w	w	w
Yfrd5-1		b	b	w	w	w

Expression of reporter genes in the wild-type strain and in mutant Yfrd strains was monitored 18 hr after colonies growing on SD-Ura plates had been overlaid with agar containing 400 μg X-Gal/ml (see materials and methods). Relative colony color: b, blue; pb, pale blue; vpb, very pale blue; and w, white.

[a] pLG312 contains a *CYC1-lacZ* reporter gene; pLG+NRE76 contains a *CYC1-*NRE^{DIT}*-lacZ* reporter gene; pLGΔSS contains a *CYC1-lacZ* reporter gene lacking a UAS; pLG+α2op contains a *CYC1-lacZ* reporter gene under the control of the α2-Mcm1 operator; pDIT1-lacZ contains a *DIT1-lacZ* translational fusion gene.

[b] Mutant Yfrd strains were identified as containing mutant alleles of *SSN6*, *TUP1*, and *SIN4* by analysis of β-galactosidase expression in diploids obtained by mating each mutant *frd* strain with strains of the opposite mating type containing *ssn6*Δ, *tup1*Δ, and *sin4*Δ alleles. Allelism of *ROX3* and *FRD13* was demonstrated by tetrad analysis of a diploid *frd13-1/ROX3-LEU2* strain.

[c] Class I mutants are defective in repression through NRE^{DIT} (NRE^{DIT-}), in repression through the α2-Mcm1 operator (α2-$Mcm1^{-}$), and in repression of a *CYC1-lacZ* reporter gene lacking a UAS ($basal^{-}$). Class II mutants are defective in repression through NRE^{DIT} (NRE^{DIT-}) and in repression through the α2-Mcm1 operator (α2-$Mcm1^{-}$), but maintain repression of a *CYC1-lacZ* reporter gene lacking a UAS ($basal^{+}$). Class III mutants appear to be specifically defective in repression through NRE^{DIT}.

holoenzyme (Gustafsson *et al.* 1997). To confirm that *frd13-1* was an allele of *ROX3*, we mated Yfrd13-1 with a wild-type strain that contained a *LEU2* marker inserted adjacent to the *ROX3* locus and analyzed tetrads derived from this diploid. In 13 of 14 tetrads analyzed, wild-type repression of the *CYC1-*NRE^{DIT}*-lacZ* reporter gene segregated with the *LEU2* marker. The other candidate genes not allelic with Class I *frd* genes were: *SPT4*, *SPT5*, and *SPT6* (Clark-Adams and Winston 1987; Neigeborn *et al.* 1987; Swanson *et al.* 1991; Swanson and Winston 1992); *SPT10* and *SPT21* (Natsoulis *et al.* 1991); *SPT16* (Malone *et al.* 1991); *BUR1* (Prelich and Winston 1993); *RGR1* (Sakai *et al.* 1990); *GAL11* (Fassler and Winston 1989; Sakurai *et al.* 1993); *MOT1* (Davis *et al.* 1992a); genes encoding histones H2A and H2B (Clark-Adams *et al.* 1988; Han and Grunstein 1988; Prelich and Winston 1993); and *RPD3* (Vidal and Gaber 1991; Vidal *et al.* 1991) (data not shown).

Class I and class II mutants are defective in repression through both NRE^{DIT} **and the α2-Mcm1 operator:** We anticipated that reduced repression through NRE^{DIT} in some of the mutants that were isolated in our screen would be due to a general defect in operator-mediated repression, particularly in Ssn6-Tup1-dependent repression. To identify at least a subset of such mutants, we monitored expression of β-galactosidase in Yfrd strains that harbored pLG+α2op, a plasmid that contains the *CYC1-lacZ* reporter gene under the control of the α2-Mcm1 operator. Repression through this well-characterized operator, which occurs in *MAT*α cells, requires the Ssn6-Tup1 corepressor (Keleher *et al.* 1992) and several other gene products, including Sin4 and Rox3 (Wahi and Johnson 1995; Carlson 1997). As expected, the

Yfrd strains containing mutations in *SSN6*, *TUP1*, *SIN4*, or *ROX3* expressed this reporter gene, as assessed by the X-Gal overlay assay (Table 3; pLG+α2op column). Additionally, Yfrd10-1, Yfrd11-1, and Yfrd12-2 expressed the α2-Mcm1 operator-containing reporter gene. We refer to those mutants that were defective in repression through both NRE^{DIT} and the α2-Mcm1 operator, but that maintained repression of the reporter gene that lacked a UAS as class II mutants (see Table 3) and concluded that they had defects in general operator-mediated repression. Wahi and Johnson (1995) also noted that *tup1* mutant cells maintain repression of basal transcription.

Candidate genes for *FRD10*, the only unidentified class II gene, included *SRB8*, *SRB9*, *SRB10*, and *SRB11*, which encode proteins that interact functionally with the carboxy terminal domain of RNA polymerase II (Kim *et al.* 1994; Koleske and Young 1994; Kuchin *et al.* 1995; Myers *et al.* 1998) and have been shown to be required for repression of *MFA2* (Wahi and Johnson 1995) or *SUC2* (Song *et al.* 1996), but not for repression of basal transcription. Plasmids containing these genes, however, failed to complement the *frd10-1* allele, indicating that *FRD10* was not one of these *SRB* genes (data not shown).

We found that the remaining five strains (Yfrd1-1, Yfrd2-1, Yfrd3-1, Yfrd4-1, and Yfrd5-1), which supported repression through the α2-Mcm1 operator (Table 1), also maintained repression of a *CYC1-lacZ* gene under the control of the URS1 operator (Vershon *et al.* 1992; data not shown). These five strains, which we refer to as class III mutants, appeared to be specifically defective in repression through NRE^{DIT}.

In summary, the mutants that we identified on the basis of defects in repression through NRE^{DIT} were placed into three different classes. Class I and class II mutants were defective in repression through the NRE^{DIT} and α2-Mcm1 operators (Table 3). Class I mutants, which were also defective in maintaining repression of a gene that lacks a UAS, included two strains with mutations in *SIN4*, one strain with a mutation in *ROX3*, and two strains with mutations in unidentified genes. Class II mutants, which maintained repression of a gene that lacks a UAS, included three strains with mutations in *SSN6*, one strain with a mutation in *TUP1*, and one strain with a mutation in an unidentified gene (Table 3). By these preliminary criteria, the five mutants of class III, which maintained repression of a gene that lacks a UAS and were effective at mediating repression through the α2-Mcm1 operator, appeared to be specifically defective in repression through NRE^{DIT}. In further studies, however, we found that Yfrd3-1 and Yfrd4-1 grew slowly in synthetic medium (data not shown). This suggested that the *FRD3* and *FRD4* genes had roles in addition to their contribution to NRE^{DIT}-mediated repression.

Effect of *frd* mutations on expression of a *DIT1-lacZ* reporter gene: Our preliminary analysis of the regulation of expression of the *DIT1* gene had suggested that there might be a component of repression that is independent of NRE^{DIT} (Friesen *et al.* 1997). This apparent NRE^{DIT}-independent repression is mediated by the sequence between NRE^{DIT} and TATA^{DIT} (Friesen *et al.* 1997). We therefore tested the *frd* strains for their ability to maintain repression of a *DIT1-lacZ* translational fusion gene that contains *DIT1* sequence from upstream of NRE^{DIT} to the initiator ATG. We have shown previously that this *DIT1-lacZ* fusion gene is repressed efficiently in wild-type cells during vegetative growth (Friesen *et al.* 1997; Table 3). We found that all class I mutants and, as expected, the three class II mutants that contained mutations in *SSN6* (Yfrd7-1, Yfrd7-2, and Yfrd7-3) were defective in repressing *DIT1-lacZ* in vegetatively growing cells (Table 3; pDIT-lacZ column). Yfrd8-1, which contained a mutant allele of *TUP1*, did not appear to be defective in repressing the *DIT1-lacZ* reporter gene (Table 3), suggesting that the *frd8-1* allele was not a null allele of *TUP1*. We previously noted that the region of *DIT1* between NRE^{DIT} and TATA^{DIT} is able to mediate *TUP1*-dependent repression (Friesen *et al.* 1997). It is therefore possible that *frd8-1* encodes a form of Tup1 that is more effective at mediating this latter repression than at mediating repression through NRE^{DIT}. *DIT1-lacZ* was also repressed in Yfrd10-1, the remaining class II mutant strain, and in the five class III mutant strains (Table 3). These latter mutants, therefore, are defective in components that contribute to NRE^{DIT}-mediated repression of a heterologous promoter, but that are not essential for repression mediated in the context of a 540-bp region from the promoter of the *DIT1* gene.

Quantification of the repression defects in the class III mutants: We next quantified the repression defects in the Class III mutants by monitoring β-galactosidase activity in cells harboring pLG+NRE76, pLG+NRE30, or pLG+NRE76 × 2/S, which contain variants of a *CYC1-lacZ* reporter gene. pLG+NRE76, the plasmid that was used to isolate the mutants, has the 76-bp NRE^{DIT}-containing fragment (nucleotides −537 to −461 of *DIT1*) inserted between the *CYC1* UAS and TATA box of the *CYC1-lacZ* reporter gene; pLG+NRE30 has a 30-bp fragment, which contains the downstream portion of NRE^{DIT} and lacks the MSE-like element (nt −493 to −464), inserted between the *CYC1* UAS and TATA box of the *CYC1-lacZ* reporter gene; and pLG+NRE76 × 2/S has two copies of the 76-bp NRE^{DIT} fragment upstream of the *CYC1* UAS of the *CYC1-lacZ* fusion gene (Friesen *et al.* 1997). The data are presented as a ratio (fold repression) of β-galactosidase activity measured from the *CYC1-lacZ* gene, which contains no negative element, to the activity measured from the *CYC1-NRE^{DIT}-lacZ* reporter gene in the same strain (Figure 2).

In the wild-type strain, expression of the *CYC1-NRE-lacZ* gene contained in pLG+NRE76 was 500-fold lower

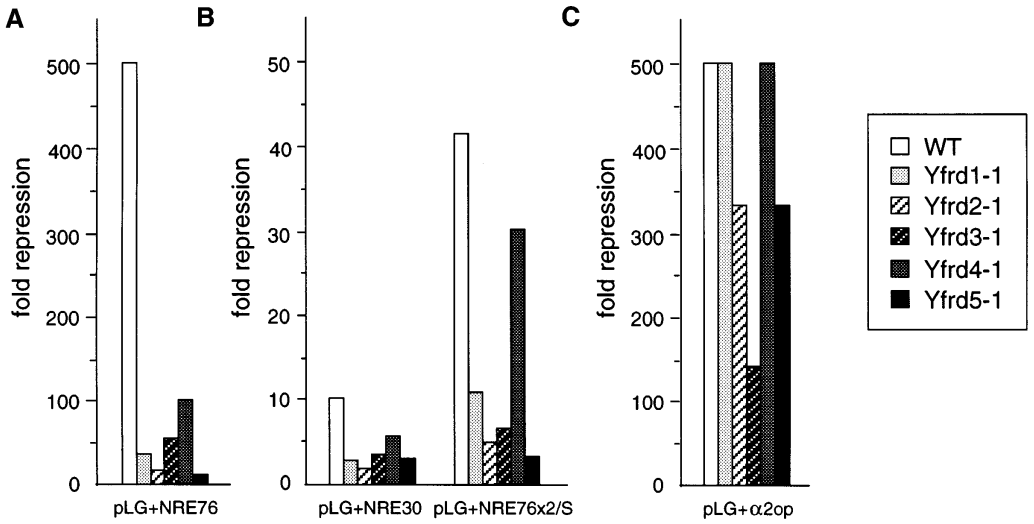


Figure 2.—Effect of class III mutations on expression of various reporter genes. Fold repression of gene expression is expressed as the ratio of β-galactosidase activity measured in cells containing the plasmid-borne *CYC1-lacZ* reporter gene to the β-galactosidase activity measured in cells of the same strain containing the indicated plasmid-borne operator-containing reporter gene. The bars indicating fold repression represent the averages of assays performed on at least three independent cultures. Cells were grown overnight to late log phase in SD-Ura, diluted, and grown for three to four generations in SD-Ura, and then harvested and assayed for β-galactosidase activity. Fold repression of gene expression (A): on insertion of NRE76 between the UAS^{CYC1} and TATA box of the *CYC1-lacZ* reporter gene; (B) on insertion of NRE30 between the UAS^{CYC1} and TATA box of the *CYC1-lacZ* reporter gene, and on insertion of two copies of NRE76 upstream of the UAS of the *CYC1-lacZ* reporter gene; and (C) on insertion of the α2-Mcm1 operator between the UAS^{CYC1} and TATA box of the *CYC1-lacZ* reporter gene. The absolute values of the β-galactosidase activities for the individual strains containing pLG312 are as follows: W303-1BT (WT), 2400 units; Yfrd1-1, 3600 units; Yfrd2-1, 1400 units; Yfrd3-1, 3100 units; Yfrd4-1, 4300 units; Yfrd5-1, 1900 units. We note that β-galactosidase activities in different strains were not assayed on the same day and so are not directly comparable between strains.

than was expression of the *CYC1-lacZ* reporter gene contained in pLG312 (Figure 2A). The 30-bp fragment containing the downstream portion of NRE^{DIT} was a much less efficient repressor element than the full 76-mer; the 30-bp fragment reduced expression of β-galactosidase 10-fold in wild-type cells (Figure 2B). As shown previously, β-galactosidase expression from pLG+NRE76 × 2/S was repressed 40-fold relative to expression of the parental reporter gene in pLG312 (Friesen *et al.* 1997; Figure 2B).

Among the class III mutant strains, Yfrd1-1, Yfrd2-1, and Yfrd5-1 were the most defective in repression through the 76-bp NRE^{DIT}-containing fragment; repression was 14- to 40-fold less efficient than in the wild-type strain (Figure 2A). The mutations in the Yfrd3-1 and Yfrd4-1 strains were less deleterious, with repression through the 76-bp-containing fragment being only 9- and 5-fold less efficient, respectively, than in the wild-type strain. This same pattern was found in repression through the 30-bp fragment representing the downstream portion of the 76-bp fragment and in repression directed by the 76-bp fragment positioned upstream of the *CYC1* UAS in the *CYC1-lacZ* reporter gene (Figure 2B). We conclude that the reduced ability of the class III mutants to mediate NRE^{DIT}-dependent repression reflects deficiencies in the contribution that the downstream portion of the 76-bp fragment makes to repression.

As a control, we also measured β-galactosidase activity in cells containing pLG+α2op. In wild-type cells, the presence of the α2-Mcm1 operator led to 500-fold repression of the reporter gene (Figure 2C). Four of the class III mutant strains (Yfrd1-1, Yfrd2-1, Yfrd4-1, and Yfrd5-1) maintained efficient repression of this reporter gene. Yfrd3-1, however, was 3-fold less efficient than the wild-type strain in mediating repression through the α2-Mcm1 operator (Figure 2C). This minor deficiency in repression through the α2-Mcm1 operator in Yfrd3-1 had escaped detection in the less sensitive X-Gal overlay assay (Table 3).

Mutation of *FRD* genes affects sporulation: We next tested the Class III mutants for their ability to form spores. Although, to date, the NRE^{DIT} element has been identified only in the promoter region of the divergently transcribed *DIT1* and *DIT2* genes, we considered it likely that this element would also regulate other as-yet-to-be-identified, mid-late sporulation-specific genes. Although we did not detect derepression of the *DIT1-lacZ* reporter gene in the class III mutants (Table 3), we speculated that inappropriate expression of some of these other hypothetical mid-late sporulation-specific genes during vegetative growth or early sporulation might lead to defects in spore formation.

Homozygous mutant *MAT***a**/*MAT*α *frd*/*frd* strains were transferred to sporulation medium, and ascus formation was monitored over a 5-day period. The efficiency of ascus formation in the wild-type strain was 62% after 40 hr in sporulation medium and 72% after 90 hr (Figure 3). The two mutant strains that grew slowly in synthetic medium, YYfrd3-1 and YYfrd4-1, were almost completely deficient in spore formation (<3% of the cells formed asci; Figure 3), and the other three class

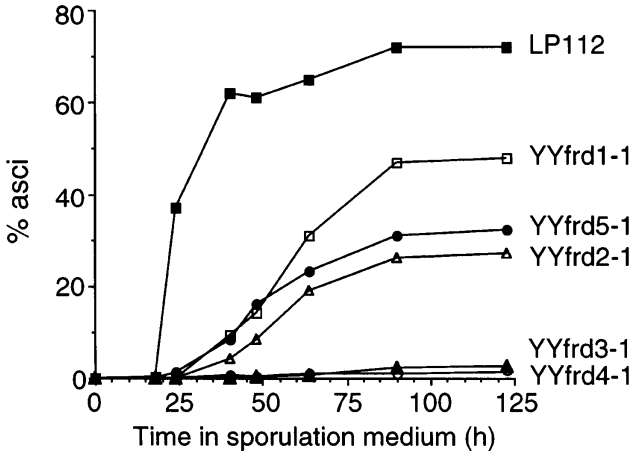


Figure 3.—Time course of ascus formation in wild-type and class III mutant strains. Wild-type diploid **a**/α cells (LP112) and diploid **a**/α cells homozygous for the indicated *frd* alleles were pregrown in SD-Ura, washed, and transferred to sporulation medium at $t = 0$. Samples were taken at various times, and the number of cells that had formed asci containing two or more spores was determined for >200 cells from each of two independent cultures for each mutant. Ascus formation is expressed as a percentage of total cells counted.

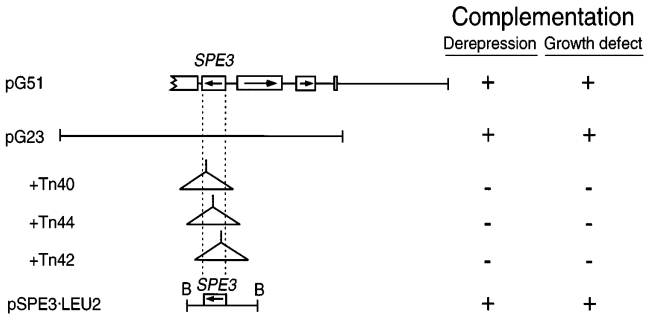


Figure 4.—Cloning *FRD3* by complementation. pG23 (containing sequences from nucleotide 678608–689892 of chromosome *XVI*, using the numbering of the Saccharomyces Genome Database) and pG51 (containing sequences from nucleotide 683257–694791), which were isolated from a yeast genomic DNA library, complemented both the derepression phenotype and the growth defect of Yfrd3-1. After pG23 had been mutagenized by Tn1000 transposon mutagenesis (Sedgwick and Morgan 1994; Morgan *et al.* 1996), plasmids containing transposon insertions were introduced into Yfrd3-1. Three plasmids that were unable to complement the derepression phenotype and the growth defect were identified and partially sequenced to determine the sites of insertion of the transposons. pG23Tn40 contained a transposon insertion at nucleotide 684740, pG23Tn42 contained a transposon insertion at nucleotide 685245, and pG23Tn44 contained a transposon insertion at nucleotide 685062. These three insertions all disrupted the open reading frame (ORF) for the *SPE3* gene, which extends from nucleotide 685432–684554. To confirm that the *SPE3* gene was responsible for complementation of the mutant phenotype, an ~2.9-kb *Bgl*II-*Bgl*II fragment that extended from nucleotide 684020–686906 and contained *SPE3* was subcloned into a *CEN ARS1* plasmid. This plasmid, pSPE3 · LEU2, complemented both the derepression phenotype and the growth defect of Yfrd3-1. Open boxes, ORFs present in the portion of the yeast insert of pG51 that overlaps with the yeast insert present in pG23; B, *Bgl*II recognition site.

III mutants, YYfrd1-1, YYfrd2-1, and YYfrd5-1, showed a delay of ~10 hr in the onset of spore formation and about a twofold reduction in the efficiency of ascus formation (Figure 3). Thus, the mutations in the strains assigned to class III led to defects that affected progression through the sporulation program.

Cloning *FRD3*: During our preliminary characterization of the class III mutants, which was carried out with cells grown on synthetic medium, we noticed that the Yfrd3-1 strain grew more slowly than did the wild-type strain (data not shown). We subsequently discovered that growing Yfrd3-1 on rich medium suppressed both its growth defect and its defect in NRE^{DIT}-mediated repression (data not shown). Growing YYfrd3-1 in rich medium (YEPA), rather than in synthetic medium, before transfer to sporulation medium also restored efficient spore formation (data not shown). The phenotypes of the other class III mutants were independent of the growth medium (data not shown). To gain insight into why the Yfrd3-1 strain had a defect in repression through NRE^{DIT} that was dependent on its growth medium, we proceeded to clone the *FRD3* gene.

Plasmids containing the *FRD3* gene were identified by transforming the original Yfrd3-1 strain with a yeast *CEN4 LEU2*-based genomic library and screening for restoration of repression of the *CYC1-*NRE^{DIT}*-lacZ* gene. Two plasmids, pG23 and pG51, that complemented both derepression of the *CYC1-*NRE^{DIT}*-lacZ* gene and the slow growth of Yfrd31 were isolated (Figure 4). Comparison of sequence obtained from the junctions of the genomic inserts with the Saccharomyces Genome Database revealed that the plasmids contained overlapping inserts from chromosome *XVI*.

To determine which of the four ORFs present in the overlapping portions of the genomic inserts of pG23 and pG51 corresponded to *FRD3*, we subjected pG23 to transposon mutagenesis and identified three plasmids that could no longer complement Yfrd3-1 (see materials and methods). Sequence analysis with primers that extended outward from the transposon (Morgan *et al.* 1996) indicated that all three insertions disrupted the ORF designated YPR069c (Figure 4). This ORF has been recently identified as the *SPE3* gene, which encodes spermidine synthase (Hamasaki-Katagiri *et al.* 1997). Consistent with this assignment of *FRD3* as *SPE3*, a low-copy plasmid that contained the *SPE3* gene complemented both the derepression and the slow growth phenotypes of Yfrd3-1 (Figure 4).

To confirm that *SPE3* was *FRD3*, and not a low-copy suppressor of the *frd3-1* mutation, we disrupted the chromosomal copy of the *SPE3* gene by integrative transformation with a DNA fragment that contained an

spe3::HISS allele. This allele contained a Tn1000 transposon with the *HIS3* gene inserted 187 nt downstream of the initiator ATG of the *SPE3* gene (see materials and methods). Both the haploid *spe3::HIS3* strain and a diploid *spe3::HIS3/frd3-1* strain were defective in NRE^{DIT}-mediated repression and growth on synthetic medium, suggesting that *FRD3* was identical to *SPE3*. We next sporulated the *spe3::HIS3/frd3-1* strain. Although we found that mutation of *SPE3* reduced spore viability, some tetrads contained four viable spores. All the progeny of 7 such tetrads and of 12 tetrads that had 2 or 3 viable spores were defective in NRE^{DIT}-mediated repression and growth on synthetic medium. We conclude that *spe3*::*HIS3* and *frd3-1* are indeed allelic.

Addition of spermidine to synthetic medium partially suppresses the *frd3* phenotype: The biosynthetic pathway for polyamines in yeast and other organisms has been determined from biochemical and genetic studies (for review see Tabor and Tabor 1984; Tabor and Tabor 1985). Spermidine synthase, the product of the *SPE3* gene, catalyzes the transfer of an aminopropyl group from decarboxylated *S*-adenosyl methionine to putrescine to give spermidine. In yeast, there is no *SPE3*-independent pathway for spermidine biosynthesis (Cohn *et al.* 1978; Hamasaki-Katagiri *et al.* 1997).

To test whether derepression of the *CYC1-NRE^{DIT}-lacZ* reporter gene in Yfrd3-1 cells grown in minimal medium was a direct effect of a deficiency of spermidine in this medium, we monitored repression of this reporter gene in cells grown in synthetic medium that had been supplemented with various concentrations of spermidine (Figure 5). Addition of spermidine to 10^{-8} m increased repression of the *CYC1-NRE^{DIT}-lacZ* reporter gene ~2-fold; addition of spermidine to 10^{-4} m, the highest concentration tested, increased repression of the *CYC1-NRE^{DIT}-lacZ* reporter gene ~10-fold. Higher concentrations of spermidine led to significant changes in the pH of the medium (data not shown) and were not tested for their effects on gene expression. Addition of spermidine to 10^{-4} m to our presporulation synthetic medium also restored ascus formation in the YYfrd3-1 strain to the wild-type level (data not shown). Addition of spermidine to the sporulation medium only, however, did not permit efficient ascus formation (data not shown).

These experiments clearly indicated that it was an absence of spermidine that led to deficient repression of the *CYC1-NRE^{DIT}-lacZ* reporter gene in the Yfrd3-1 strain and to the sporulation defect in YYfrd3-1. In contrast, the two- to threefold defect in repression through the α2-Mcm1 operator that we had observed in the Yfrd3-1 strain grown in minimal medium (Figure 2C) was not suppressed by spermidine (Figure 5). It is possible that exogenous spermidine was required at a concentration higher than 10^{-4} m to correct for this latter defect.

Phenotype of a *spe3::HIS3* allele: We next compared the phenotype of Yfrd3-1 with the phenotype of

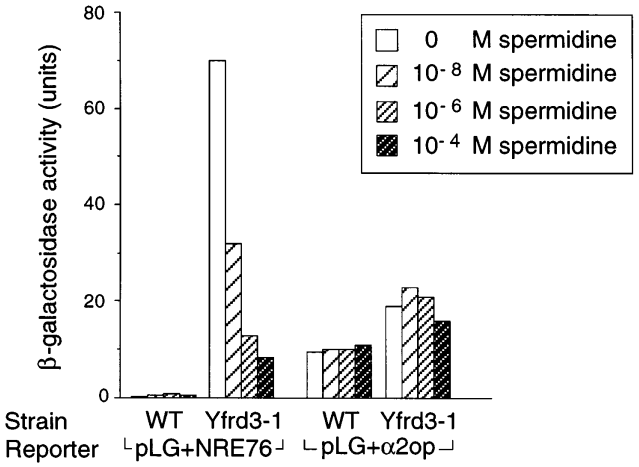


Figure 5.—Addition of spermidine to the growth medium suppresses the derepression phenotype of Yfrd3-1. Cells of strains W303-1BT (denoted WT) and Yfrd3-1 containing the indicated plasmids were grown overnight in SD-Ura containing various concentrations of spermidine, and then diluted in the same medium grown for three to four generations, harvested, and assayed for β-galactosidase activity. β-Galactosidase activities are averages of assays performed on at least three independent cultures. pLG+NRE76 and pLG+α2op contain a *CYC1-lacZ* reporter gene with NRE^{DIT} and the α2-Mcm1 operator, respectively, inserted between the *CYC1* UAS and TATA box.

Yspe3::HIS3α, a strain that contained a disrupted *spe3::HIS3* allele (see above). Yspe3::HIS3α was viable on minimal medium although, like Yfrd3-1, it grew more slowly than did the wild-type strain. The plasmid-borne *CYC1-NRE^{DIT}-lacZ* reporter gene was derepressed to the same extent in Yfrd3-1 and Yspe3::HIS3α grown in minimal medium; NRE^{DIT}-mediated repression was 43-fold in the mutant cells *vs.* 350-fold in wild-type cells (Figure 6A). Overall, therefore, the mutant strains were ~8-fold less efficient than the wild-type strain at mediating repression through NRE^{DIT}. Supplementing the medium with spermidine restored NRE^{DIT}-mediated repression in the mutant strains to within threefold of the level of repression observed in the wild-type strain (Figure 6A). Similar results were obtained on examination of expression of an integrated *CYC1-NRE^{DIT}-lacZ* reporter gene; NRE^{DIT}-mediated repression was 22- and 31-fold in Yfrd3-1 and Yspe3::HIS3α, respectively, *vs.* 110-fold in the wild-type strain (Figure 6B). Addition of spermidine to the medium reduced expression of the integrated reporter gene in both mutant strains to the same low level as in the wild-type strain (Figure 6B). Because the extent of derepression of the *CYC1-NRE^{DIT}-lacZ* gene in Yspe3::HIS3α was no greater than in Yfrd3-1, we conclude that the *frd3-1* allele was a null allele.

In these experiments, we found that the *frd3-1* and *spe3::HIS3* alleles led to a modest increase in expression of our control *CYC1-lacZ* reporter gene in cells grown in minimal medium; this increase was suppressed by

A High-copy plasmid-borne reporter gene

Units β-galactosidase activity

Genotype	reporter gene: *CYC1-lacZ*		*CYC1-NRE^{DIT}-lacZ*		Fold-repression	
	−	+	−	+	−	+
WT	2000	2000	5.7	6.8	350	290
frd3-1	5500	2900	130	29	43	100
spe3::HIS3	4000	2200	94	10	43	220

B Single-copy integrated reporter gene

Units β-galactosidase activity

Genotype	reporter gene: *CYC1-lacZ*		*CYC1-NRE^{DIT}-lacZ*		Fold-repression	
	−	+	−	+	−	+
WT	180	210	1.6	1.5	110	140
frd3-1	770	260	35	1.8	22	140
spe3::HIS3	830	440	27	1.4	31	310

Figure 6.—Expression of *CYC1-lacZ* and *CYC1-NRE^{DIT}-lacZ* genes in wild-type and *frd3* strains. (A) β-Galactosidase activity was measured in cells of strains W303-1BT (WT), Yfrd3-1, and Yspe3::HIS3α harboring, as indicated, a *CYC1-lacZ* reporter gene on a high-copy plasmid or a *CYC1-NRE^{DIT}-lacZ* reporter gene on a high-copy plasmid. The data in the − column are from cells that had been grown overnight in SD-Ura with no exogenous spermidine, diluted, and grown for three to four generations in the same medium before being harvested and assayed for β-galactosidase activity. The data in the + column are from cells grown as described above, but in SD-Ura that contained 10^{-4} m spermidine. Units of β-galactosidase activity are given as the averages of assays performed on at least three independent cultures. Fold-repression refers to the effect of the NRE on the activity of the *CYC1* UAS in the indicated strain and in the indicated growth medium; *i.e.*, the β-galactosidase activity of a strain containing pLG312 was divided by the β-galactosidase activity of the same strain containing pLG+NRE76 and grown in the same medium. (B) β-Galactosidase activity was measured as described above in cells of strains W303-1BT (WT), Yfrd3-1, and Yspe3::HIS3α that contained, as indicated, a single-copy, integrated version of the *CYC1-lacZ* reporter gene or a single-copy, integrated version of the *CYC1-NRE^{DIT}-lacZ* reporter gene.

addition of spermidine to the medium (Figure 6). We note that throughout this study we have reported the efficiency of repression relative to expression of the control *CYC1-lacZ* reporter gene in the *same* strain; thus, the changes in repression that we present as fold-effects reflect changes in NRE^{DIT} activity only.

In summary, we uncovered *SPE3* (*FRD3*) as a gene that is required for efficient repression through NRE^{DIT}, but is dispensable for repression of basal transcription. In our preliminary characterization of the mutant *FRD* strains, we had classified Yfrd3-1 as a class III mutant because it appeared to be specifically defective in NRE^{DIT}-mediated repression. We have reassigned Yfrd3-1 to the class II group of mutants, however, because we noted that Yfrd3-1, in addition to a conditional slow-growth phenotype, had general defects in gene expression. Mutation of *SPE3* (*FRD3*) not only led to less efficient repression through NRE^{DIT} but also caused a two- to threefold reduction in repression through the α2-Mcm1 operator and a two- to threefold increase in expression of our control *CYC1-lacZ* reporter gene when cells were grown in minimal medium. Because some of these defects could be partially suppressed by the addition of spermidine to the medium, we conclude that one role of spermidine may be to modulate gene expression.

DISCUSSION

In this study, we have further characterized NRE^{DIT}-mediated repression. This negative element directs Ssn6-Tup1-dependent repression of the sporulation-specific *DIT1* and *DIT2* genes in vegetative cells (Friesen *et al.* 1997). We have demonstrated that *SIN4* is required to achieve full repression of a *CYC1-NRE^{DIT}-lacZ* reporter gene. NRE^{DIT} thus becomes the third Ssn6-Tup1-dependent element that is known to require *SIN4* for full repression. *SIN4*, which encodes a component of the RNA polymerase II holoenzyme (Li *et al.* 1995), has been shown previously to contribute to the Ssn6-Tup1-dependent repression of *MFA2* (Chen *et al.* 1993) and *SUC2* (Song *et al.* 1996), as well as to repression and activation of a number of Ssn6-Tup1-independent genes (Jiang and Stillman 1992; Chen *et al.* 1993; Covitz *et al.* 1994). Because both Sin4 (Jiang and Stillman 1992; Jiang *et al.* 1995; Macatee *et al.* 1997) and the Ssn6-Tup1 complex (Roth *et al.* 1992; Cooper *et al.* 1994; Gavin and Simpson 1997) have been implicated in modulation of chromatin structure, we consider it likely that NRE^{DIT}-mediated repression occurs, at least in part, by regulation of chromatin structure.

Three classes of *frd* mutants: To gain further insight into the mechanism of NRE^{DIT}-mediated repression, we isolated mutants that were defective in repression of a *CYC1-NRE^{DIT}-lacZ* reporter gene. We tentatively assigned these *FRD* (function in repression of *DIT*) mutants, which represented 12 complementation groups, to three classes. We note that although some genes were isolated more than once, this screen was not saturating.

Class I mutants, in which basal transcription was increased, included strains with mutations in *SIN4* and *ROX3*/*SSN7* and two strains with mutations in unidentifed genes. *ROX3*, which encodes a component of the mediator complex of RNA polymerase II holoenzyme (Gustafsson *et al.* 1997), has been shown to play a role in repression of three other genes regulated by Ssn6-Tup1: *CYC7* (Rosenblum-Vos *et al.* 1991), *SUC2* (Song *et al.* 1996), and *MFA2* (Carlson *et al.* 1997). We note that previous studies of *rox3*/*ssn7* mutants did not test for a defect in repression of basal transcription. Class II, which consisted of mutants that had defects in operator-mediated repression but maintained repression of basal transcription, included strains with mutations in *SSN6* and *TUP1* and one strain with a mutation in an unidentified gene.

Mutant strains that appeared to be specifically defec-

tive in NRE^{DIT}-mediated repression were assigned to class III. These strains are good candidates for having a mutation in a gene(s) encoding an NRE^{DIT}-binding protein(s). We found that mutation of the class III *FRD* genes caused only a partial loss of repression through NRE^{DIT}. It is possible that these genes encode proteins that do not have a key role in establishing a repression complex or that these *frd* alleles are not null alleles. The incomplete defects in repression seen for the class III *FRD* mutants could also reflect partial functional redundancies among the class III *FRD* gene products.

Identification of *FRD3* as *SPE3*: A major finding of this study was the demonstration that *FRD3* is identical to *SPE3*, the gene encoding spermidine synthase. *SPE3* has been cloned recently as a gene complementing the spermidine auxotrophy of a *spe3-1* mutant strain (Hamasaki-Katagiri *et al.* 1997). Although our preliminary characterization of Yfrd3-1 had suggested that the *frd3-1* mutation specifically affected NRE^{DIT}-mediated repression of gene expression, further study indicated that Yfrd3-1 had additional deficiencies, including a conditional slow-growth phenotype and minor defects in expression of other genes. To our knowledge, *SPE3* has never before been identified through its effects on gene expression.

In contrast to the report by Hamasaki-Katagiri *et al.* (1997) that an *spe3*Δ mutant is unable to grow on synthetic medium to which no spermidine has been added, we found that an *spe3::HIS3* mutant was able to grow, albeit slowly, on such medium. This discrepancy could be due to the presence of trace amounts of spermidine in our synthetic medium, but not in that used by Hamasaki-Katagiri *et al.* (1997) (Balasundaram *et al.* 1991). Alternatively, it is possible that our *spe3::HIS3* allele allowed synthesis of a truncated, but partially active, enzyme.

Role for spermidine in modulating gene expression: Spermidine is the predominant polyamine in yeast with intracellular concentrations in the millimolar range (Cohn *et al.* 1978). Extensive studies have shown that polyamines are essential for optimal growth in all cell types and implicate them as contributors to processes such as DNA replication, transcription, translation, protein phosphorylation, and resistance to elevated temperature and oxygen toxicity, among other things (Balasundaram *et al.* 1993; Balasundaram *et al.* 1996; for review see Tabor and Tabor 1984, Tabor and Tabor 1985, Davis *et al.* 1992b). Nonetheless, the molecular role of spermidine *in vivo* remains to be defined. *In vitro*, polyamines have been shown to bind to DNA and RNA (Igarashi *et al.* 1982), to condense DNA (Marx and Reynolds 1982), and to enhance the binding of some proteins to DNA and to inhibit the binding of others (Panagiotidis *et al.* 1995).

We have found that growth in minimal medium of yeast cells that cannot synthesize spermidine leads to defects in gene expression. The most dramatic defect that we observed was in NRE^{DIT}-mediated repression: mutation of *SPE3* (*FRD3*) led to an ~8-fold reduction in repression of a *CYC1-NRE^{DIT}-lacZ* gene reporter (Figure 2A, Figure 6A). Additionally, we found that *spe3* (*frd3*) mutants expressed a *CYC1-lacZ* reporter gene at a two to threefold higher level than did wild-type cells and were two- to threefold less efficient than were wild-type cells in mediating repression through the α2-Mcm1 operator. Both the defect in repression through NRE^{DIT} and the overexpression of the *CYC1-lacZ* gene were partially suppressed by the addition of spermidine to the growth medium. Thus, the elevated expression of the *CYC1-NRE^{DIT}-lacZ* reporter gene in *spe3* (*frd3*) cells grown in minimal medium may be the combined effect of a defect in repression through NRE^{DIT} and a defect in modulating the activity of the *CYC1* UAS. We note that in this study we have reported the efficiency of NRE^{DIT}-mediated repression relative to expression of the control *CYC1-lacZ* reporter gene in the same strain; thus, the fold-effects that we refer to reflect changes in NRE^{DIT} activity only. Our data, therefore, clearly indicate that the predominant effect of spermidine on restoring repression to the *CYC1-NRE^{DIT}-lacZ* reporter gene in an *spe3* strain is through its effects on NRE^{DIT}.

Spermidine could act to modulate gene expression in various ways. Its effect could be indirect; spermidine-induced changes in processes such as translational fidelity (Balasundaram *et al.* 1994) might lead to differential synthesis of regulators of transcription. Spermidine could modulate gene expression directly by affecting the binding of sequence-specific DNA-binding proteins to their cognate sites on DNA. Indeed, Panagiotidis *et al.* (1995) demonstrated that *in vitro* spermidine enhances the binding of several proteins to DNA and inhibits the binding of others. It is also possible that spermidine promotes an interaction between the Ssn6-Tup1 corepressor and the NRE^{DIT}-binding protein. Future identification of the NRE^{DIT}-binding protein(s) will allow us to test for these potential roles of spermidine in regulating assembly of a repression complex at NRE^{DIT}.

Spermidine, which has a polybasic character similar to that of histones, could also modulate gene expression by promoting localized changes in DNA structure. Indeed, *in vitro*, spermidine binds to DNA and promotes its compaction (Marx and Reynolds 1982). It is possible, therefore, that *in vivo* spermidine acts in conjunction with nucleosomes to reduce differentially the accessibility of regions of DNA to regulators of transcription and to the general transcription machinery. This effect could prevent hyperactivation of positively regulated genes (such as *CYC1*), as well as lead to more efficient repression of negatively regulated genes (such as *DIT1*). In this case, the absence of spermidine would lead to higher levels of gene expression by allowing the transcriptional machinery readier access to promoter regions. In support of this model, polyamines have been

found to increase the stability of nucleosome core particles *in vitro* (Morgan *et al.* 1987). Furthermore, chromatin from HeLa cells depleted of polyamines by treatment with inhibitors shows increased accessibility to DNase (Snyder 1989). It has been suggested that regulation of polyamine binding to DNA could be achieved through acetylation and deacetylation of polyamines (Matthews 1993) in a manner similar to the way histone acetylation and deacetylation regulate the association of nucleosomes with DNA (for review see Pazin and Kadonaga 1997; Wolfe 1997; Struhl 1998). Finally, our data indicate that the absence of spermidine does not affect expression of all genes to the same extent; this is also true for mutations in histones and many general regulators of transcription.

In summary, we have identified 12 *FRD* genes that contribute to NRE^{DIT}-mediated repression. These *FRD* genes include *SSN6*, *TUP1*, *SIN4*, *ROX3*, and *SPE3*. Our identification of *SPE3*, which encodes spermidine synthase, as a modulator of gene expression provides support for an *in vivo* role for spermidine, be it direct or indirect, in the regulation of gene expression. Further characterization of the *FRD* mutants that we have identified in this study, as well as isolation of additional *FRD* genes, will lead to a better understanding of the mechanism by which NRE^{DIT} represses gene expression.

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