MOLECULAR AND CELLULAR BIOLOGY, Jan. 1997, p. 123–134 Vol. 17, No. 1
0270-7306/97/$04.00+0

An Ssn6-Tup1-Dependent Negative Regulatory Element Controls Sporulation-Specific Expression of *DIT1* and *DIT2* in *Saccharomyces cerevisiae*

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Received 19 June 1996/Returned for modification 30 July 1996/Accepted 9 October 1996

Sporulation of the yeast *Saccharomyces cerevisiae* is a process of cellular differentiation that occurs in *MAT*a/*MAT*α diploid cells in response to starvation. The sporulation-specific genes *DIT1* and *DIT2*, which are required for spore wall formation, are activated midway through the sporulation program, with maximal transcript accumulation occurring at the time of prospore enclosure. In this study, we have identified a negative regulatory element, termed NRE^{DIT}, that is located between the start sites of transcription of these divergently transcribed genes. This element, which prevents expression of the *DIT1* and *DIT2* genes during vegetative growth, reduces expression of a *CYC1-lacZ* reporter gene more than 1,000-fold and acts in an orientation- and position-independent manner. We found that the ability of NRE^{DIT} to turn off expression of the reporter gene and the chromosomal *DIT1* and *DIT2* genes in vegetative cells requires the Ssn6-Tup1 repression complex. Interestingly, NRE^{DIT}-mediated repression of the reporter gene is maintained during sporulation. Derepression during sporulation requires complex interactions among several *cis*-acting elements. These are present on an ~350-bp DNA fragment extending from NRE^{DIT} to the TATA box and an ~125-bp fragment spanning the TATA box of *DIT1*. Additionally, a region of NRE^{DIT} which is very similar in sequence to UAS^{SPS4}, an element that activates gene expression midway through sporulation, contributes both to vegetative repression and to sporulation-specific induction of *DIT1*. We propose a model to explain the requirement for multiple elements in overcoming NRE^{DIT}-mediated repression during sporulation.

Sporulation of the yeast *Saccharomyces cerevisiae* is a process of cellular differentiation that begins when *MAT*a/*MAT*α diploid cells are starved for nitrogen in the absence of a fermentable 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. First, the starved cell exits the cell cycle and completes a single round of DNA replication. 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 wall formation initiates at the spindle pole bodies. A double membrane, which is generated by the coalescence of lipid vesicles, forms around each of the four haploid meiotic products. Ultimately each daughter nucleus, as well as cytoplasmic material, is engulfed within a prospore. Deposition of spore wall material then generates a multilayered spore wall and gives rise to four mature spores within the ascal sac (reviewed in references 9 and 18). Progression through the sporulation program depends on the expression of at least four temporally distinct groups of sporulation-specific genes, classified as early, middle, mid-late, and late based on their time of expression (reviewed in reference 30). Sporulation in *S. cerevisiae* therefore provides a model system to study the temporal control of gene expression during development.

Early meiotic genes, which are involved in events such as pairing of homologous chromosomes, recombination, and segregation of chromosomes, appear to be regulated by proteins binding to URS1 and UAS sites (reviewed in reference 30). The URS1 element, which was first identified as a site mediating repression of the arginase gene, has now been found in the promoter regions of many genes (28). This element has a dual role in regulating expression of early meiotic genes: URS1 mediates repression of these genes during mitotic growth and acts in conjunction with a UAS element to mediate their activation during meiosis (4, 8, 52). Characterization of the promoter elements of the early meiotic genes *HOP1* and *IME2* has led to the identification of two activation elements, referred to as UAS_H and T_4C, respectively (4, 52). Sequence inspection has shown that a URS1 site and either a UAS_H or T_4C site are present in the upstream regions of nearly all early meiotic genes (reviewed in reference 30).

Some of the proteins that are involved in mediating the URS1- and UAS-dependent regulation of early meiotic genes have been identified. Binding of Ume6, a protein with a C6-type Zn cluster DNA-binding domain, to the URS1 site leads to repression in mitotic cells and contributes to activation during meiosis (1, 4, 5, 34, 45, 46). Recent studies have shown that at the onset of sporulation, Ime1, a key activator of early meiotic genes, binds to Ume6, converting it from a negative to a positive regulator (5, 40, 45). This modified Ume6 then acts in conjunction with a nearby UAS site, such as T_4C or UAS_H, to activate gene expression (4, 5, 8, 52). A factor that interacts with UAS_H in both vegetative and sporulating cells (37) has been shown to be Abf1 (19).

Expression of early meiotic genes is followed by the sequential expression of middle, mid-late, and late genes. Because the times of expression of these genes have not yet been compared in a common genetic background, particularly for postmeiotic sporulation-specific genes, these temporal classifications should

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be considered provisional. The regulatory regions of several middle and late genes have been characterized. A 15-bp region, designated UAS^{SPS4}, which is located close to the TATA box of the middle sporulation-specific gene *SPS4*, recently has been shown to confer sporulation-specific expression on a heterologous reporter gene (22). This UAS element is remarkably similar to a sequence within the regulatory region of the sporulation-specific *SPR2* gene (11, 36). Because a factor that binds to UAS^{SPS4} is present in vegetative cells, Hepworth et al. (22) speculated that during sporulation this factor is modified to serve as an activator of the *SPS4* gene or, alternatively, that the factor recruits an activator to the *SPS4* promoter. The promoter of another postmeiotic sporulation-specific gene, *SPR3*, contains an Abf1-binding site that is essential for its sporulation-specific expression pattern (33). Putative Abf1-binding sites have been found in the upstream regions of many sporulation-specific genes (19, 33). Expression of the late sporulation-specific gene *SGA1* has been shown to depend on two sequence elements which have not yet been characterized in detail: a UAS element which responds to nutritional control and a negative element which responds to both mating-type control and nutritional control (26). The divergently transcribed late sporulation-specific genes *SPS18* and *SPS19* appear to be regulated by a common UAS (12).

We have initiated an analysis of the regulatory region of the divergently transcribed genes *DIT1* and *DIT2*. These genes, which are the only identified members of the class of mid-late sporulation-specific genes, encode enzymes that are required for biosynthesis of the dityrosine precursor that is incorporated into the outermost layer of the spore wall (6, 7). In this work, we present evidence that repression of the *DIT1* and *DIT2* genes during vegetative growth depends on the Ssn6-Tup1 repression complex. 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 (17, 25, 31, 56, 57). Genetic and biochemical evidence indicates that Ssn6 and Tup1, neither of which appears to bind DNA, associate in a complex that is recruited to the promoters of coordinately regulated genes by pathway-specific DNA-binding proteins (25, 49, 50, 54; reviewed in references 39 and 47). DNA-binding proteins that mediate this regulation include α2 for cell-type-specific genes (25, 44), Mig1 for glucose-repressed genes (48), and presumably Rox1 and Crt1 for oxygen-regulated and DNA damage-inducible genes, respectively (2, 14, 56). Current evidence indicates that Ssn6-Tup1 may repress transcription by affecting the general transcription machinery (23) and by causing alterations in chromatin structure (13, 15, 29).

In this work we show that Ssn6-Tup1-dependent repression of the *DIT1* and *DIT2* genes during vegetative growth is mediated by a potent negative regulatory element (NRE), referred to as NRE^{DIT}. NRE^{DIT} prevents expression of a reporter gene fused to a heterologous promoter; however, this repression is maintained during sporulation. We found that overcoming Ssn6-Tup1-mediated repression of *DIT1* during sporulation involves complex interactions among several *cis*-acting regulatory elements.

MATERIALS AND METHODS

Yeast strains. The diploid *MAT***a**/*MAT*α strains of *S. cerevisiae* used in this study were obtained by mating the isogenic haploid strains W3031A (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and W3031B (*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) or their derivatives W3031A-H (*MAT***a** *ade2-1 leu2-3,112 trp1-1 ura3-1 can1-100*) and W3031B-T (*MAT*α *ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100*). The strain obtained by mating W3031A and W3031B is referred to as LP112; the strain obtained by mating W3031A-H and W3031B-T is referred to as LP112-HT. The Δ*dit1* Δ*dit2::URA3* allele was obtained as follows. First, a 4.8-kb *Bam*HI-*Sal*I fragment, which spans the *DIT1* and *DIT2* genes, was isolated from pPB10 (7); the *Bam*HI site is located at the 3′ end of the *DIT2* gene, and the *Sal*I site is located in vector sequence adjacent to the 3′ end of the *DIT1* gene. This fragment was cloned into pEMBL19. Two contiguous *Bal*I fragments, which contain the sequence encoding the first 269 amino acids of the *DIT1* gene product, the *DIT1-DIT2* intergenic region, and the sequence encoding the first 294 amino acids of the *DIT2* gene product, were then replaced with a 1.1-kb *Hin*dIII-*Hin*dIII fragment containing the *URA3* gene. The *Hin*dIII-generated ends were filled in with the Klenow form of DNA polymerase prior to ligation. The resultant plasmid, pPB44E, was digested with *Hin*dIII and *Eco*RI to give a DNA fragment containing the *URA3* gene flanked on one side with sequence from the 3′ end of *DIT1* and flanked on the other side with sequence from the 3′ end of *DIT2*. This fragment was used to transform the haploid strains W3031A-H and W3031B-T. Appropriate integrative transformation was confirmed by Southern blot analysis of genomic DNA from Ura$^+$ transformants. A *MAT***a** Δ*dit1* Δ*dit2::URA3* strain and a *MAT*α Δ*dit1* Δ*dit2::URA3* strain were then mated to give the diploid strain LP112-HTΔdit1Δdit2.

The haploid strains used to examine the role of *SSN6* and *TUP1* were EG123 (*MAT***a** *SSN6 TUP1 trp1 leu2 ura3 his4*) and derivatives thereof containing either the *ssn6Δ9* allele (42) or the Δ*tup1::LEU2* allele (25). These strains were provided by A. Johnson.

Media and culture conditions. Media were as described previously (22). Sporulation medium contained 1% potassium acetate plus the required auxotrophic supplements. For sporulation, yeast strains were grown in minimal medium (SD) to ~10^7 cells per ml. Cells were then diluted into presporulation medium (YEPA), grown for ca. three generations, washed, and transferred to sporulation medium at a density of ~10^7 cells per ml. All yeast cultures were grown at 30°C. The time of transfer of cells to sporulation medium is referred to as 0 h. The efficiency of ascus formation was 30 to 65% as assessed by light microscopy. EG123-derived strains were grown in SD to ~10^7 cells per ml and then diluted into rich medium (YEPD) and grown for ca. three generations.

Deletions into the 5′-flanking regions of the *DIT1* and *DIT2* genes. pSH-DIT1+900, which was used to make unidirectional deletions extending towards *DIT1*, was constructed by first cloning a 2.1-kb *Hin*dIII-*Sal*I fragment containing the coding sequence of *DIT1* between the corresponding sites of pRS315 (43). An ~900-bp *Hin*dIII-*Hin*dIII fragment containing the *DIT1-DIT2* intergenic region was then inserted in the appropriate orientation at the *Hin*dIII site of this plasmid to recreate the *DIT1* gene with its upstream sequence. pPB51H, which was used to make deletions extending towards *DIT2*, was constructed as follows. First, the *DIT1* gene was removed from pPB10 by religation of pPB10 DNA that had been digested with *Nar*I. A 2.1-kb *Eco*RI-*Hin*dIII fragment, which contains the *DIT2* gene with 400 bp of 5′-flanking sequence and ~100 bp of pBR322 sequence (from *Sal*I to *Nar*I), was isolated from the resultant plasmid, cloned between the corresponding sites of pEMBL18, and then recovered as a 2.4-kb *Pvu*II-*Pvu*II fragment. This fragment was cloned into *Pvu*II-digested pRS315 to give pPB51H. The control plasmid pSH-DIT1+2 consists of a 4.8-kb *Sal*I-*Bam*HI fragment containing the *DIT1* and *DIT2* genes cloned into the corresponding sites of pRS315.

Unidirectional deletions into the 5′-flanking sequence of *DIT1* and *DIT2* were obtained with pSH-DIT1+900 that had been digested with *Sac*I and *Bam*HI and pPB51H that had been digested with *Pst*I and *Sal*I, respectively, by using an Erase-a-Base exonuclease III kit (Promega). Deletion endpoints were determined by dideoxy sequence analysis (41) of double-stranded DNA with the −20 universal primer. In this paper, plasmids containing *DIT1* and *DIT2* are named p(-X)DIT1 and p(-X)DIT2, respectively. In both instances, X denotes the position of the deletion endpoint, in nucleotides, from the start site of transcription of the *DIT1* gene.

Construction of plasmids to test fragments for negative regulatory activity. *DIT1*-derived sequences were assayed for their effect on expression of the *CYC1-lacZ* reporter gene present in pLGΔ312(Bgl) (provided by A. Mitchell), a derivative of pLGΔ312 (20). Throughout this paper the reporter gene on this plasmid is referred to as *CYC1*(*UAS*)-*lacZ*. pLGΔ312(Bgl) contains two unique restriction sites into which fragments may be inserted to test for NRE activity: a *Bgl*II site (which is flanked by *Sal*I and *Xho*I sites) located at nucleotide (nt) −178, between the *CYC1* UASs and TATA box, and a *Sma*I site located at nt −312, upstream of the UASs.

pLG312+NRE76 and pLG312+NRE69 were constructed from pLG+24o, pLG+22o, and pLG+22F as follows. An ~200-bp *Pvu*II-*Nar*I fragment from p(−537)DIT1 was cloned into the *Bgl*II site of pLGΔ312(Bgl) to give pLG+22F. The *Bgl*II-generated ends of pLGΔ312(Bgl) had been end filled with the Klenow form of DNA polymerase prior to ligation. The *Pvu*II-*Nar*I fragment contains 121 bp of vector sequence (derived from pBluescript II KS; Stratagene) fused to 76 bp of *DIT1* (nt −537 to −462; +1 is the start site of transcription of *DIT1*). The *DIT1* sequence is in the same orientation, referred to as the forward orientation, with respect to the TATA box of the *CYC1*(*UAS*)-*lacZ* gene as it is in the *DIT1* gene. Complementary oligonucleotides containing 5′-GATC overhangs were annealed and cloned into the *Bgl*II site of pLGΔ312(Bgl) to give pLG+24o and pLG+22o. These plasmids contain *DIT1* sequence from nt −537 to −494 and from nt −530 to −502, respectively. For construction of the plasmids pLG312+NRE76 and pLG312+NRE69, we took advantage of a unique *Bal*I site at nt −505 of the *DIT1* sequence and a unique *Sac*I site in the *lacZ* sequence of

the plasmids described above. pLG312+NRE76 was constructed by ligating the ~8-kb *Sac*I-*Bal*I fragment from pLG+24o with the ~1-kb *Bal*I-*Sac*I fragment from pLG+22F. pLG312+NRE69 was constructed in the same way by ligating the ~8-kb *Sac*I-*Bal*I fragment from pLG+22o with the ~1-kb *Bal*I-*Sac*I fragment from pLG+22F. This regenerates pLGΔ312(Bgl) with *DIT1* sequence inserted at the *Bgl*II site without any adjacent pBluescript sequence. Thus, pLG312+NRE76 contains *DIT1* sequence from nt −537 to −462 and pLG312+NRE69 contains *DIT1* sequence from nt −530 to −462.

pLG312+NRE76/S and pLG312+2xNRE76/S were constructed by first subcloning a 90-bp *Sal*I-*Sal*I fragment from pLG312+NRE76 into the *Sal*I site of pBluescript II KS to make pBST+NRE76. One or two copies of a 90-bp *Hinc*II fragment (containing *DIT1* sequences from nt −537 to −462) from this plasmid were then cloned into the *Sma*I site of pLGΔ312(Bgl), generating pLG312+NRE76/S and pLG312+2xNRE76/S, respectively. pLG-NRE76 was constructed in the same manner as was pLG+22F but contains the ~200-bp insert in the opposite orientation. This plasmid therefore contains 121 bp of vector DNA in addition to *DIT1* sequence inserted at the *Bgl*II site of pLGΔ312(Bgl). Control experiments showed that the pBluescript-derived sequence alone had no NRE activity (data not shown).

pLG312+NRE53 was made by extending the annealed oligonucleotides NRE45B (5′-AGAGAACCCTTTTATTTATGGCCAGACGCGCGTCGCAAAAAAGG-3′) and NRE22T (5′-GATGTTTACCTTTTTTTGCGAC-3′) with the Klenow form of DNA polymerase and then cloning the double-stranded fragment into the *Bgl*II site of pLGΔ312(Bgl) which had been end filled with the Klenow form of DNA polymerase. pLG312+NRE53 contains a single insert in the same orientation with respect to the *CYC1* TATA box as it is with respect to the *DIT2* TATA box.

pLG312+NRE44 was made in two steps. First, an ~45-bp *Bal*I-*Nar*I fragment containing *DIT1* sequences from nt −505 to −462 was end filled with the Klenow form of DNA polymerase and ligated with pBluescript that had been digested with *Eco*RV and *Sma*I to give pBST+NRE44. Second, an ~400-bp PCR product was amplified from pBST+NRE44 by using extended versions of the universal and reverse sequencing primers (5′-CGTTGTAAAACGACGGCCAGT-3′ and 5′-CAGCTATGACCATGATTACGC-3′, respectively; the extensions are underlined). This PCR product was digested with *Xba*I and *Hin*dIII and cloned in the *Bgl*II site of pLGΔ312(Bgl) after all ends had been filled in with the Klenow form of DNA polymerase to give pLG312+NRE44.

pLG312+HH was made in two steps. First, an ~480-bp *Hin*fI-*Hin*fI fragment from p(−537)DIT1-lacZ, containing 26 bp of vector-derived sequence fused to the *DIT1* sequence from nt −537 to −76 downstream, was end filled with the Klenow form of DNA polymerase and inserted into the *Sma*I site of pBluescript II KS to give pBST+HH. Next, an ~500-bp *Kpn*I-*Sac*I fragment from pBST+HH was cloned into the *Bgl*II site of pLGΔ312(Bgl), after all ends had been blunted by treatment with the Klenow form of DNA polymerase.

The sequences and orientations of all inserts in the pLGΔ312(Bgl) fusion genes were verified by dideoxy sequence analysis of double-stranded DNA.

Construction of plasmids to test fragments for UAS activity. pLGΔ312SΔSS (Bgl) (provided by A. Mitchell) is a derivative of pLGΔ312S (21) in which the *Sma*I-*Sal*I fragment containing the *CYC1* UASs has been replaced with a *Bgl*II linker, creating a unique restriction site into which fragments may be inserted to test for UAS activity. Throughout this paper the gene on this plasmid is referred to as *CYC1-lacZ*. pLGΔSS+NRE76 was constructed by cloning the *Sal*I-*Sal*I fragment from pLG312+NRE76, which contains *DIT1* sequence from nt −537 to −462, into the *Sal*I site of pLGΔ312SΔSS(Bgl). To make pLGΔSS+NRE29, complementary oligonucleotides with GATC overhangs, corresponding to *DIT1* sequence from nt −530 to −502, were annealed and cloned into the *Bgl*II site of pLGΔ312SΔSS(Bgl). pLGΔSS+NH was made by subcloning a *Nar*I-*Not*I fragment from pBST+HH, which contains *DIT1* sequence from nt −463 to −76, into the *Bgl*II site of pLGΔ312SΔSS(Bgl) after all ends had been end filled with the Klenow form of DNA polymerase. pLGΔSS+HH was made by subcloning a *Kpn*I-*Sac*I fragment from pBST+HH, which contains *DIT1* sequence from nt −537 to −76, into the *Bgl*II site of pLGΔ312SΔSS(Bgl) after all ends were blunted with the Klenow form of DNA polymerase. The sequences and orientations of all inserts into pLGΔ312ΔSS(Bgl) were verified by dideoxy sequence analysis of double-stranded DNA.

Construction of *DIT1-lacZ* translational fusions. All plasmids containing *DIT1-lacZ* translational fusions were derived from pRY64 (provided by Roger Yocum), a 2μm-based *URA3*-containing plasmid with a unique *Bam*HI site introduced just downstream of the initiator methionine of the *lacZ* gene. Fusion genes were created by introducing restriction fragments containing *DIT1* sequence between the *Bam*HI and *Sma*I sites of pRY64. *DIT1* sequences were obtained from an ~600-bp PCR product amplified from p(−537)DIT1 by using as the downstream primer an oligonucleotide (BamHI-ATG-DIT1 [5′-CCC GGATCCATTTTTGTTAATGTCTCGACAA-3′]) that had a *Bam*HI site at the 5′ end (underlined) adjacent to the *DIT1* ATG (double underlined). The 5′ end of the upstream primer NRE22T (see above) was at nt −537 of *DIT1*. A fragment obtained by digestion of the PCR product with *Bam*HI was treated with T4 polynucleotide kinase and used to generate p(−537)DIT1-lacZ, a fragment obtained by digestion of the PCR product with *Bal*I and *Bam*HI was used to generate p(−505)DIT1-lacZ, and a fragment obtained by digestion of the PCR product with *Nar*I was end filled with the Klenow form of DNA polymerase and then digested with *Bam*HI and used to generate p(−463)DIT1-lacZ.

An ~200-bp PCR product was amplified from p(−114)DIT1 by using the BamHI-ATG-DIT1 primer described above as the downstream primer and the extended version of the universal sequencing primer as the upstream primer. This PCR product was used to make p(−114)DIT1-lacZ, p(−76)DIT1-lacZ, and pNRE(−114)DIT1-lacZ. To make p(−114)DIT1-lacZ, the PCR product was digested with *Bam*HI, treated with T4 polynucleotide kinase, and cloned between the *Bam*HI and *Sma*I sites of pRY64. To make p(−76)DIT1-lacZ, the PCR product was digested with *Hin*fI, end filled with the Klenow form of DNA polymerase, digested with *Bam*HI, and cloned between the *Bam*HI and *Sma*I sites of pRY64. The PCR product was also treated with T4 polynucleotide kinase and ligated, without any restriction enzyme treatment, into the blunted *Kpn*I site of pBST-NRE76 to give pBST-NRE(−114 to ATG). An ~300-bp *Eco*RV-*Bam*HI fragment from pBST-NRE(−114 to ATG) that contained NRE^{DIT} (nt −537 to −462) adjacent to the *DIT1* sequence from nt −114 to +53 was then cloned between the *Bam*HI and *Sma*I sites of pRY64 to give pNRE(−114)DIT1-lacZ. The sequences of the promoter regions of the modified pRY64 plasmids were verified by dideoxy sequence analysis of double-stranded DNA.

RNA isolation and Northern analysis. RNA was prepared from yeast and analyzed by Northern blotting with gene-specific probes as described previously (22, 35) by using 15 μg of total RNA per lane. The *DIT2* probe was prepared with a 2.1-kb *Eco*RI-*Hin*dIII fragment from pPB51H.

β-Galactosidase assays. β-Galactosidase activity was measured in extracts of cells prepared from 5-ml cultures grown in YEPD, YEPA, or sporulation medium as described previously (22) except that cells of strain EG123 and its derivatives were washed in 20 mM Tris-Cl (pH 7.5)–10 mM EDTA (50) to disrupt clumps before freezing. The activities reported are averages obtained from three to six cultures. We repeated each experiment two to four times, and we consistently found that the relative levels of β-galactosidase activities were similar from one experiment to the next. β-Galactosidase activity is given in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein at 28°C. We used a plate assay (3) to compare the levels of expression of reporter genes in LP112 cells growing on acetate and on glucose. We found that the carbon source did not affect the relative levels of expression.

RESULTS

Expression of *DIT1* is controlled by an NRE upstream of nt −463. As a first step in identifying the regulatory element(s) promoting sporulation-specific expression of *DIT1*, we mapped the 5′ boundary of the regulatory region of the gene. For these experiments, we monitored the effects of sequential deletions of 5′-flanking sequence on expression of *DIT1* present on a plasmid. The *DIT1*-containing plasmids were introduced into a *MAT***a**/*MAT*α Δ*dit1*/Δ*dit1* Δ*dit2*/Δ*dit2* strain. Chromosome-derived *DIT* transcripts are not present in this strain (Fig. 1A, lanes 1 and 10) because the *DIT1* and *DIT2* genes have been deleted. RNA isolated from cells harvested during vegetative growth and 12 h after transfer to sporulation medium was examined for the expression of plasmid-borne *DIT1* genes by Northern blotting. First, we confirmed that sporulation-specific expression of both *DIT1* and *DIT2* was maintained from a plasmid containing the entire *DIT1-DIT2* region (Fig. 1A, lanes 2 and 11). *DIT1* was also expressed in a sporulation-specific manner from a plasmid containing 866 bp of 5′-flanking sequence (Fig. 1A, lanes 3 and 12). In contrast, a *DIT1* gene containing 463 bp of 5′-flanking sequence (where +1 is the start site of transcription) was expressed both during vegetative growth and during sporulation, with the level of transcript accumulation during vegetative growth being similar to that during sporulation (Fig. 1A, lanes 4 and 13). We found that *DIT1* genes containing 300, 128, and 114 bp of 5′-flanking sequence were expressed at similar levels during vegetative growth and sporulation (Fig. 1A, lanes 5, 7, 8, 14, 16, and 17). Expression from a *DIT1* gene with a deletion endpoint at nt −192 was unusual in that transcript accumulation was much lower in sporulating cells than in vegetative cells (Fig. 1A, lanes 6 and 15). Finally, expression of a *DIT1* gene with a deletion endpoint at nt +26, which lacked the predicted TATA box and start site of transcription, was barely detectable (Fig. 1A, lanes 9 and 18). These results suggested that a negative regulatory element whose 5′ boundary was between nt −866 and −463 prevented expression of *DIT1* in vegetative cells. The data also

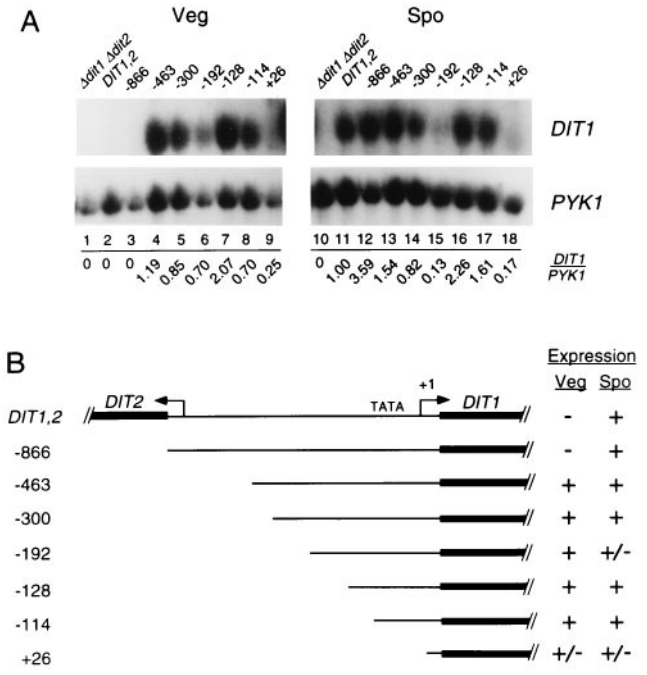

FIG. 1. The 5′ boundary of the regulatory region of the *DIT1* gene is located between nt −866 and −463. (A) Northern blot analysis was performed with RNAs isolated from LP112-HTΔdit1Δdit2 (*MAT***a**/*MAT*α *Δdit1 Δdit2::URA3*/*Δdit1 Δdit2::URA3*) (lanes 1 and 10), from LP112-HTΔdit1Δdit2 containing pSH·DIT1+2 (lanes 2 and 11), and from LP112-HTΔdit1Δdit2 containing versions of pDIT1 with the deletion endpoint of *DIT1* denoted above each lane (+1 is the transcriptional start site) (lanes 3 to 9 and lanes 12 to 18) (see Materials and Methods). RNA was isolated from cells growing in YEPA (Veg) and from cells harvested 12 h after transfer to sporulation medium (Spo). The filters were hybridized sequentially with radioactively labeled probes containing *DIT1* and *PYK1*. The ratios of the intensities of *DIT1* and *PYK1* signals were obtained by PhosphorImager analysis with IPLabGel software. These ratios were normalized to the ratio obtained for cells containing pSH·DIT1+2 during sporulation (*DIT1,2* [lane 11]), which was arbitrarily set at 1.0, and are given below the gels. (B) Summary of the data of panel A with schematic representations of the *DIT1* genes analyzed. The deletion endpoints of the plasmid-borne *DIT1* genes are indicated on the left. *DIT1,2* indicates that the plasmid contained *DIT1* and *DIT2*. +, *DIT1* transcripts readily detectable (normalized *DIT1*/*PYK1* ratio, >0.7); +/−, reduced level of *DIT1* transcripts (normalized *DIT1*/*PYK1* ratio, 0.13 to 0.25); −, *DIT1* transcripts not detected.

indicated that the 50-bp sequence upstream of the putative TATA box, which is located between nt −64 and −59, sufficed to promote a significant level of *DIT1* expression during vegetative growth and during sporulation. We have previously shown that the vector sequence present upstream of the plasmid-borne *DIT1* gene does not contain UAS activity (22).

Repression of *DIT1* and *DIT2* during vegetative growth is controlled by a regulatory element located between nt −537 and −485. To delimit the upstream boundary of the NRE controlling expression of *DIT1*, we analyzed expression from plasmid-borne *DIT1* genes with deletion endpoints in the region between nt −866 and −463. As assessed by Northern blot analysis, we found that *DIT1* genes with deletion endpoints at nt −857, −772, −748, −605, and −537 were expressed only in sporulating cells, whereas *DIT1* genes with deletion endpoints at nt −502 and −483 were expressed at comparable levels during vegetative growth and during sporulation (data not shown). This indicated that the upstream boundary of a putative NRE that prevented expression of *DIT1* during vegetative growth was between nt −537 and −502.

Because the transcriptional start sites of the divergently transcribed and coordinately activated *DIT1* and *DIT2* genes are only 828 bp apart, we investigated whether the NRE that acted on *DIT1* might also regulate expression of *DIT2*. To test this possibility, we examined expression of plasmid-borne *DIT2* genes that contained various amounts of 5′-flanking sequence. The results of Northern blot analysis of transcript accumulation from these *DIT2* genes are summarized in Fig. 2. We first monitored expression of a plasmid-borne *DIT2* gene that contained 365 bp of 5′-flanking sequence. This sequence, which ended at nt −462 with respect to the transcriptional start site of the *DIT1* gene, would be expected to include the negative regulatory site that we had identified as acting on *DIT1*. We found that this *DIT2* gene was expressed in a sporulation-specific manner (Fig. 2). We next tested plasmid-borne *DIT2* genes that had more extensive deletions for sporulation-specific expression. For consistency, the deletion endpoints are defined in this paper relative to the start site of transcription of the *DIT1* gene. *DIT2* expression from genes with deletion endpoints at nt −465 and −485 remained sporulation specific. In contrast, genes with deletion endpoints at nt −491, −494, −521, and −523 were expressed in vegetatively growing cells. Transcript accumulation from the genes with deletion endpoints at nt −491 and −494 was not as high during vegetative growth as during sporulation, whereas transcript accumulation from genes with deletion endpoints at nt −521 and −523 was similar during vegetative growth and sporulation. These results suggested that a common element prevented vegetative expression of both *DIT1* and *DIT2*. The *DIT1*-proximal boundary of this element was between nt −485 and −491, and the *DIT2*-proximal boundary was between nt −537 and −502.

NRE^{DIT} represses a gene driven by a heterologous UAS. The results of the deletion analyses described above suggested that a sequence between nt −485 and −537 of the *DIT1* gene contained an NRE. As a first test for NRE activity in this region, we inserted a 76-bp fragment, extending from nt −537 to −462 of *DIT1*, between the UAS and TATA box of a plasmid-borne *CYC1*(*UAS*)-*lacZ* reporter gene (see Materials and Methods). The presence of this fragment, which we refer to as NRE^{DIT}, led to a 1,000-fold reduction in expression of

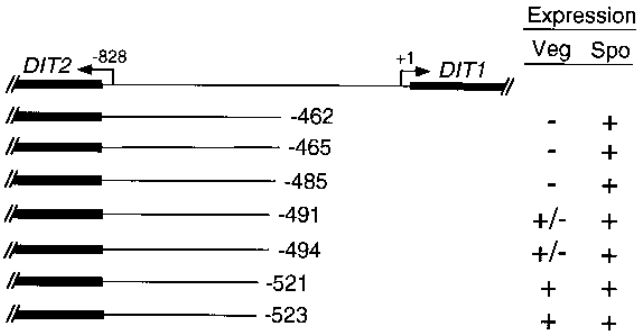

FIG. 2. An NRE also acts on *DIT2*. Schematic representations for data from a Northern blot analysis of RNAs from strain LP112-HTΔdit1Δdit2 containing versions of pDIT2 (see Materials and Methods) are given. The position of the deletion endpoints are denoted with the numbering in nucleotides from the start site of transcription of *DIT1*. For example, the deletion endpoint −462 is 365 nt upstream of the start site of transcription of *DIT2*. RNA was isolated from cells growing vegetatively in YEPA (Veg) and from cells harvested 12 h after transfer to sporulation medium (Spo). A Northern filter was hybridized sequentially with radioactively labeled probes containing *DIT1* and *PYK1*, and the ratios of the intensities of the *DIT2* signal to the *PYK1* signal were obtained by PhosphorImager analysis with IPLabGel software. These ratios were normalized to the ratio obtained for p(−462)DIT2 during sporulation, which was arbitrarily set at 1.0. +, transcripts readily detectable (normalized *DIT1*/*PYK1* ratio, 0.5 to 3); +/−, transcript level significantly reduced (normalized *DIT1*/*PYK1* ratio, 0.3 to 0.5); −, little or no transcript detectable (normalized *DIT1*/*PYK1* ratio, <0.1).

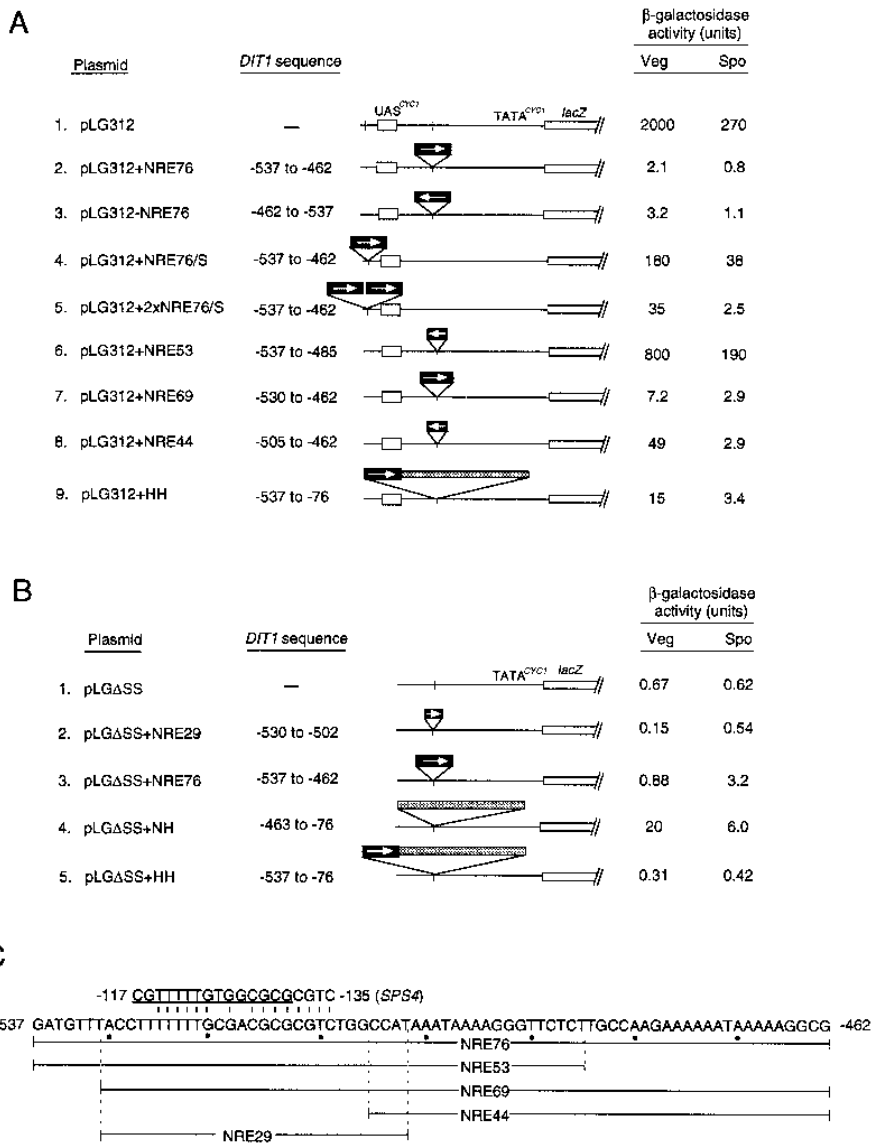

FIG. 3. The *DIT1* sequence from nt −530 to −462 contains an NRE. The effect of *DIT1* sequence on expression of a *CYC1-lacZ* reporter gene containing the *CYC1* UASs (A) and lacking the *CYC1* UASs (B) was monitored. (A) The *CYC1*(*UAS*)-*lacZ* reporter gene of pLGΔ312(Bgl) is shown in line 1. This plasmid contains a unique *Bgl*II site between the *CYC1* UASs and TATA box and a unique *Sma*I site upstream of the *CYC1* UASs. Plasmids which contained a region of the *DIT1* upstream sequence inserted into the *Bgl*II site (lines 2 and 3 and 6 to 9) or the *Sma*I site (lines 4 and 5) of pLGΔ312(Bgl) (see Materials and Methods) were transformed into the diploid strain LP112. β-Galactosidase activity in extracts of cells harvested during vegetative growth in YEPA (Veg) and 12 h after transfer to sporulation medium (Spo) was measured. Numbering is from the *DIT1* start site of transcription. Black boxes, *DIT1* sequence from nt −537 to −462 (referred to in the text as NRE^{DIT}), or a portion thereof, with the arrow pointing toward the *DIT1* gene indicating the orientation of the insert (see Materials and Methods); shaded rectangles, *DIT1* sequence from nt −463 to −76 (referred to in the text as the middle fragment); pLG312, pLGΔ312(Bgl). (B) The *CYC1-lacZ* reporter gene of pLGΔ312SΔSS(Bgl), which contains no UASs, is shown in line 1. The indicated *DIT1* sequences were inserted into the unique *Bgl*II site of this plasmid (see Materials and Methods). Data are presented as described for panel A. pLGΔSS refers to pLGΔ312SΔSS(Bgl). (C) Sequence upstream of *DIT1*, from nt −537 to −462, referred to in this paper as NRE^{DIT}. The horizontal bars below the sequence denote fragments from this region that were tested for regulatory activity. The top sequence is the bottom strand of *SPS4* from nt −117 to −135. Identities with *DIT1* sequence are noted with vertical lines, and the sequence referred to as UAS^{SPS4} is underlined.

the *CYC1*(*UAS*)-*lacZ* reporter gene during vegetative growth as assessed by measuring β-galactosidase activity in extracts of plasmid-containing cells (Fig. 3A, compare lines 1 and 2). This fragment also prevented expression of the reporter gene when inserted in the opposite orientation (Fig. 3A, line 3; see Materials and Methods) and when present upstream of the *CYC1* UAS (Fig. 3A, lines 4 and 5). In the latter case, two copies of the NRE gave more efficient repression than one. Interestingly, we found that the repressive effect of this fragment on expression of the reporter gene was not relieved during sporulation (Fig. 3A, compare Veg and Spo columns).

In an attempt to further delimit the boundaries of the NRE, we assayed smaller fragments for NRE activity (Fig. 3C). A 53-bp fragment, which encompassed the region defined by deletion analysis as being required for negative regulation (nt −537 to −485) (Fig. 3C, NRE53), had minimal NRE activity (Fig. 3A, line 6), whereas a 69-bp fragment that extended from nt −530 to −462 retained NRE activity (Fig. 3A, line 7; Fig.

3C, NRE69). Hepworth et al. (22) noted that the *DIT1* sequence from nt −525 to −513 contained an 11-of-13-bp match with UAS^{SPS4}, an element that activates sporulation-specific expression of the middle sporulation-specific gene *SPS4* (Fig. 3C). If adjacent sequence is taken into consideration, the match is 15 of 17 bp. We found that a 44-bp fragment that extended from nt −505 to −462, and therefore lacked this UAS^{SPS4}-like element, retained a significant level of NRE activity, leading to a 40-fold reduction in expression of the reporter gene during vegetative growth and a 60-fold reduction during sporulation (Fig. 3A, line 8; Fig. 3C, NRE44). This indicated that the UAS^{SPS4}-like element in NRE^{DIT} is not required for NRE activity, although this sequence, or adjacent sequence, does appear to make a minor contribution to negative regulation.

In summary, we found that NRE^{DIT} was a potent NRE. This element reduced expression of a reporter gene more than 1,000-fold and acted in an orientation- and position-independent manner. The finding that NRE^{DIT}-mediated repression of a heterologous gene was retained during sporulation suggested that additional *DIT*-derived sequences were necessary to permit expression of *DIT1* during sporulation.

NRE^{DIT} has minimal UAS activity during sporulation. We next examined the possibility that the 76-bp NRE-containing fragment might contain separate negative and positive regulatory elements and that the UAS^{SPS4}-related sequence might act as a UAS when removed from a neighboring site(s). We tested the activity of a 29-bp *DIT1*-derived fragment (Fig. 3C, NRE29) that contained the UAS^{SPS4}-related sequence in the context of a *CYC1*(*UAS*)-*lacZ* reporter gene and a *CYC1-lacZ* reporter that lacked a UAS. [In this paper, we distinguish between a *CYC1-lacZ* reporter gene that contains the *CYC1* UAS and one that does not by referring to a *CYC1*(*UAS*)-*lacZ* reporter gene and a *CYC1-lacZ* reporter gene, respectively.] The fragment had neither NRE activity, as assessed with the *CYC1*(*UAS*)-*lacZ* reporter gene (data not shown), nor UAS activity, as assessed with the *CYC1-lacZ* reporter gene, during vegetative growth and during sporulation (Fig. 3B, compare lines 1 and 2).

We also tested the possibility that a low level of sporulation-specific activation mediated by NRE^{DIT} might have been undetectable in the presence of the strong *CYC1* UAS of the reporter gene used for the experiment whose results are shown in Fig. 3A (line 2). We inserted the 76-bp fragment containing NRE^{DIT} into the *CYC1-lacZ* reporter gene. We found that this fragment did not support expression of *CYC1-lacZ* during vegetative growth (Fig. 3B, compare lines 1 and 3). A very low level of expression, however, could be detected during sporulation.

Because our initial deletion analysis had revealed that *DIT1* was expressed in a sporulation-specific manner in the absence of any sequence upstream of NRE^{DIT} (Fig. 2A), it seemed likely that a UAS located between the NRE and the TATA box was responsible for expression during sporulation. Indeed, we found that the *DIT1* fragment extending from the downstream boundary of NRE^{DIT} (nt −463) to 13 bp upstream of the TATA box (nt −76), which we refer to as the middle fragment, had modest UAS activity both in vegetative cells and during sporulation (Fig. 3B, line 4). We next tested whether the middle fragment contained a *cis*-acting element which could relieve NRE^{DIT}-mediated repression during sporulation. Insertion of both NRE^{DIT} and the middle fragment into the *CYC1*(*UAS*)-*lacZ* reporter gene did not lead to derepression during sporulation (Fig. 3A, line 9). Similarly, insertion of both NRE^{DIT} and the middle fragment into the *CYC1-lacZ* reporter gene did not permit activation of this gene during sporulation (Fig. 3B, line 5).

In summary, these data showed that although NRE^{DIT} alone had negative regulatory activity and the downstream middle fragment (nt −463 to −76) alone had positive regulatory activity, together these sequences were not sufficient to promote sporulation-specific expression of a heterologous reporter gene.

Requirements for sporulation-specific expression. Because the *DIT1* fragment from nt −537 to −76 did not promote expression of the *CYC1-lacZ* reporter gene during sporulation (Fig. 3B, line 5), we tested whether the sequence at the start site of transcription was required to overcome the effect of the NRE during sporulation. We first constructed a translational fusion gene containing *DIT1* sequence from nt −537 to the initiator methionine codon (nt +53) fused to the *lacZ* coding region in a vector similar to that used for construction of the *CYC1-lacZ* transcriptional fusion genes. This reporter gene, which we refer to as (*−537*)*DIT1-lacZ*, was expressed in a sporulation-specific manner (Fig. 4A and B, lines 1). The level of β-galactosidase activity in sporulating cells was 200-fold greater than that in vegetatively growing cells. This suggested that the *DIT1* sequence between nt −76 and +53, which encompasses the putative TATA box and start site of transcription, provided an element that was essential for expression during sporulation in a gene regulated by NRE^{DIT}.

We next tested whether NRE^{DIT} and a fragment spanning the start site of transcription of the *DIT1* gene would generate sporulation-specific expression. Reporter genes containing *DIT1* sequence from nt −114 to +53 [(*−114*)*DIT1-lacZ*] or from nt −78 to +53 [(*−78*)*DIT1-lacZ*] supported constitutive expression of β-galactosidase (Fig. 4A, lines 2 and 4). We found that insertion of NRE^{DIT} abolished expression of (*−114*)*DIT1-lacZ* both in vegetatively growing cells and during sporulation (Fig. 4A, line 3). Thus, transcription from the fragment spanning the start site of transcription of the *DIT1* gene remained sensitive to NRE^{DIT}-mediated repression during sporulation. In summary, we found that neither a reporter gene containing NRE^{DIT} and *DIT1* upstream sequence from nt −463 to −76 (Fig. 3B, line 5) nor a reporter gene containing NRE^{DIT} and *DIT1* sequence from nt −114 to +53 (Fig. 4A, line 3) was derepressed during sporulation. We therefore concluded that sporulation-specific expression depends on at least three *cis*-acting sequences: NRE^{DIT}, an element present in the middle fragment (nt −463 to −114), and an element present in the fragment spanning the start site of transcription (nt −76 to +53) which we refer to as the start-site-containing fragment.

Redundant control of sporulation-induced expression. We next examined the effect of deleting NRE^{DIT} from the sporulation-specific translational fusion gene (*−537*)*DIT1-lacZ*. As expected, we found that the reporter gene (*−463*)*DIT1-lacZ*, which lacked NRE^{DIT}, was expressed in vegetative cells (Fig. 4B, line 3). However, β-galactosidase activity from this fusion gene was consistently higher in sporulating cells than in cells growing vegetatively (18 versus 3.6 U in the experiment shown). This observation prompted us to compare expression of (*−463*)*DIT1-lacZ* and (*−537*)*DIT1-lacZ* as cells progressed through sporulation. β-Galactosidase activity from (*−537*)*DIT1-lacZ* was negligible in vegetatively growing cells (Fig. 4C). Enzyme activity began to increase between 8 and 10 h after transfer of cells to sporulation medium, reaching a maximal level that was 1,000 times greater than the background level measured in vegetatively growing cells (Fig. 4C). The (*−463*)*DIT1-lacZ* translational fusion gene was induced during sporulation with the same kinetics as was seen for (*−537*)*DIT1-lacZ*, with the maximal level of activity being approxi-

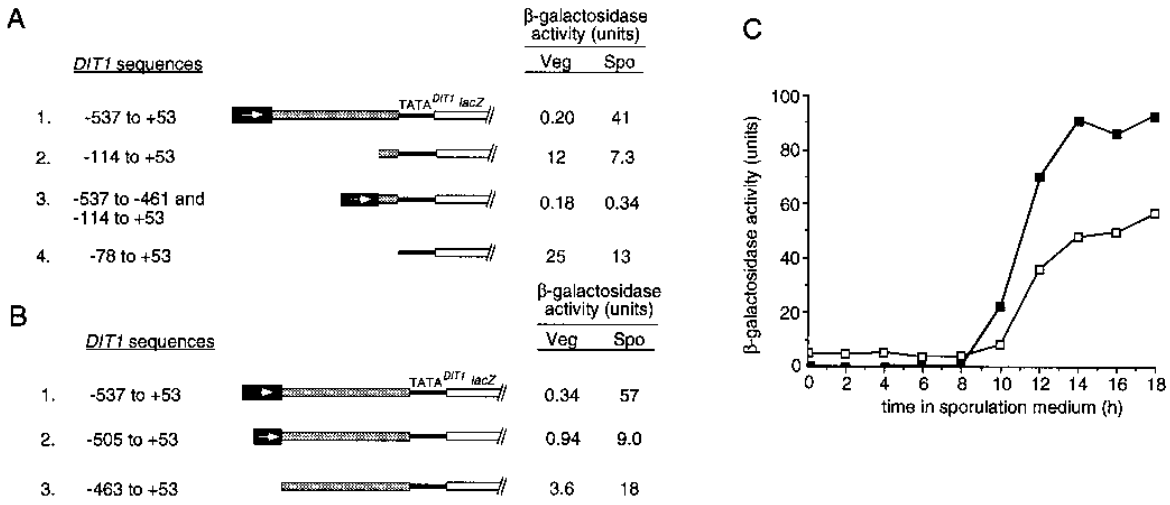

FIG. 4. Activities of *DIT1-lacZ* translational fusion genes during vegetative growth and sporulation. (A) Derepression of *DIT1* during sporulation requires three regions of DNA. The indicated *DIT1* sequences, which all extended to the initiator ATG (nt +53) of *DIT1*, were fused to the *lacZ* gene present in pRY64 (see Materials and Methods), and expression of the fusion genes was monitored as described in the legend to Fig. 3. The black lines in the schematic diagram of the fusion genes, denoted TATA^{DIT1}, represent the *DIT1* sequence from nt −78 to the initiator ATG at +53. Other symbols are as described for Fig. 3. The plasmids used for the experiments were p(−537)DIT1-lacZ (line 1), p(−114)DIT1-lacZ (line 2), pNRE(−114)DIT1-lacZ (line 3), and p(−78)DIT1-lacZ (line 4). (B) The UAS^{SPS4}-like element of NRE^{DIT} is required for high levels of sporulation-specific activation. The indicated *DIT1* sequences, which all extended to the initiator ATG (+53) of *DIT1*, were fused to the *lacZ* gene present in pRY64 (see Materials and Methods), and expression of the fusion genes was monitored as described in the legend to Fig. 3. (C) Expression of *DIT1-lacZ* fusion genes during sporulation. Strain LP112 containing p(−537)DIT1-lacZ (closed squares) or p(−463)DIT1-lacZ (open squares) was grown in YEPA and transferred to sporulation medium. β-Galactosidase activity in samples of cells harvested at 2-h intervals after transfer to sporulation medium was measured.

mately one-half that of (*−537*)*DIT1-lacZ* (Fig. 4C). However, the ratio of the activity in sporulating cells to the activity in vegetative cells was only 12 for (*−463*)*DIT1-lacZ*, whereas it was 1,000 for (*−537*)*DIT1-lacZ*. This difference in the extent of induction was due both to a higher level of expression of (*−463*)*DIT1-lacZ* than (*−537*)*DIT1-lacZ* during vegetative growth and to a lower level of expression during sporulation. In summary, these data showed that the *DIT1* sequence extending from the 3′ boundary of NRE^{DIT} to the ATG (nt −463 to +53) could confer modest sporulation-specific induction. We concluded that NRE^{DIT} contributes to sporulation-specific expression of *DIT1* by providing strong repression during vegetative growth and by making a minor contribution to activation during sporulation (also see below).

We also examined expression from a translational fusion gene, (*−505*)*DIT1-lacZ*, that contained *DIT1* sequence from nt −505 to +53. This gene retained a partially functional NRE (Fig. 3A, line 8) but lacked the UAS^{SPS4}-like element. As expected, the level of β-galactosidase activity observed in vegetative cells containing (*−505*)*DIT1-lacZ* was less than that observed in cells containing (*−463*)*DIT1-lacZ*, which lacked NRE^{DIT}, but was more than that observed in cells containing (*−537*)*DIT1-lacZ*, which contained NRE^{DIT} (Fig. 4B). Expression during sporulation from (*−505*)*DIT1-lacZ*, however, was consistently lower than expression from (*−537*)*DIT1-lacZ* (Fig. 4B, compare lines 1 and 2, and data not shown). We concluded, therefore, that the UAS^{SPS4}-like element makes a minor contribution both to repression in vegetative cells and to activation during sporulation.

***SSN6* and *TUP1* are required for repression through NRE^{DIT}.** We next tested whether the Ssn6-Tup1 repression complex, which prevents transcription of sets of coregulated genes, was involved in repression of *DIT1* and *DIT2* during vegetative growth. The Ssn6-Tup1 complex does not bind DNA on its own; rather, it is recruited to promoters by interactions with family-specific DNA-binding proteins: α2 for cell-type-specific genes, Mig1 for glucose-repressed genes, and presumably Rox1 for hypoxic genes and Crt1 for DNA-damage-inducible genes (reviewed in references 39 and 47).

To test whether repression by NRE^{DIT} required Ssn6 and Tup1, we monitored expression of *CYC1*(*UAS*)*-lacZ* reporter genes containing NRE^{DIT} in *ssn6* and *tup1* haploid strains. We note that the strains used for this series of experiments, which are derived from EG123, are not related to our standard laboratory strain, referred to as LP112, which is a diploid derived from mating isogenic W3031 strains (see Materials and Methods). The EG123-derived strains supported a higher level of basal transcription of *CYC1-lacZ* (for example, we see 18-fold higher levels of β-galactosidase activity in EG123 cells containing the *CYC1-lacZ* reporter gene than in LP112 cells containing this gene [Fig. 5B, line 1, and Fig. 3B, line 1]). Ssn6 and Tup1 are known to affect expression of *CYC1* (reviewed in references 38 and 55). Indeed, mutation of *SSN6* or *TUP1* led to a 10-fold decrease in expression of the *CYC1*(*UAS*)*-lacZ* reporter gene (Fig. 5A, line 1).

We found that both *SSN6* and *TUP1* were essential for repression through NRE^{DIT}. NRE^{DIT} reduced expression of the *CYC1*(*UAS*)*-lacZ* reporter gene in the wild-type EG123 strain 10,000-fold (Fig. 5A, compare lines 1 and 2, *WT* column). The NRE, however, had no effect on expression of the reporter gene in isogenic *ssn6* and *tup1* strains during vegetative growth (Fig. 5A, compare lines 1 and 2, *ssn6* and *tup1* columns). Similarly, we found that NRE^{DIT} was not able to repress the *CYC1-lacZ* reporter gene in **a**/α diploid *ssn6*/*ssn6* or **a**/α diploid *tup1*/*tup1* cells during vegetative growth (data not shown). (We did not assay **a**/α diploid cells after transfer to sporulation medium because *ssn6* and *tup1* strains are unable to sporulate [10, 53].) Thus, Ssn6 and Tup1 are required for repression through NRE^{DIT}.

Because our experiments had suggested that NRE^{DIT} contributed to activation of *DIT1* during sporulation, we tested whether the NRE had UAS activity in *ssn6* or *tup1* strains. We

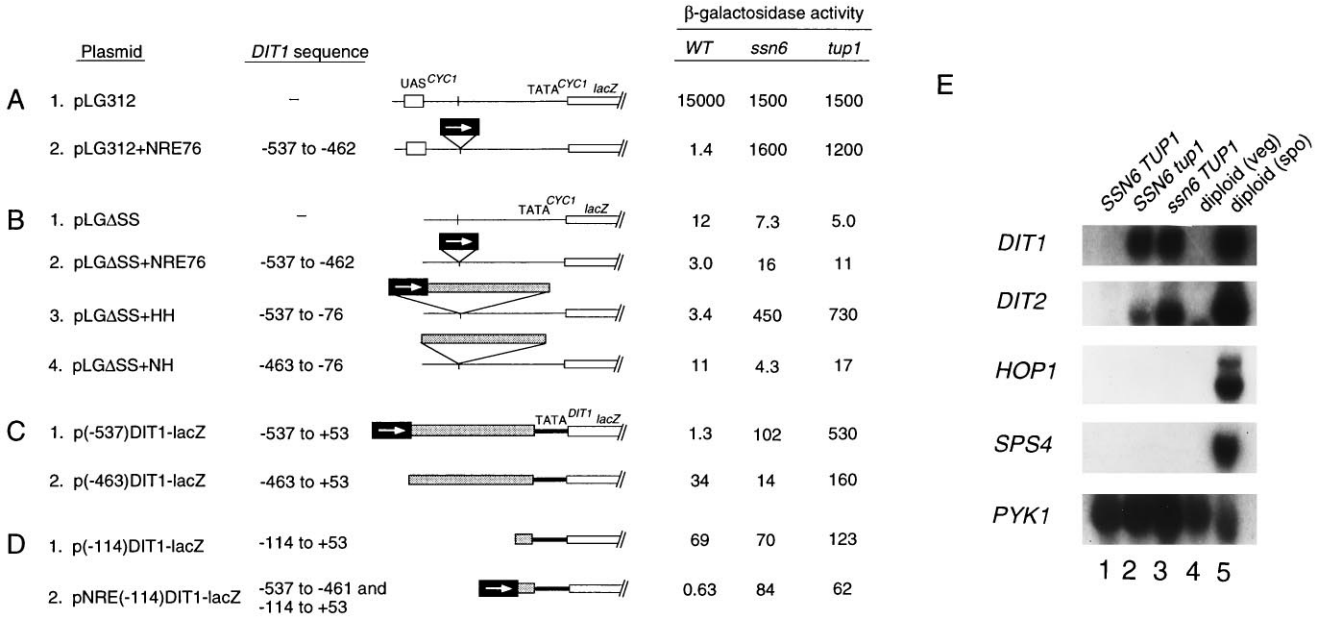

FIG. 5. Analysis of gene expression in wild-type, *ssn6*, and *tup1* cells. Symbols are as described for Fig. 3. (A) Repression by NRE^{DIT} requires Ssn6 and Tup1. pLGΔ312(Bgl) and pLG312+NRE76 were transformed into wild-type EG123 (*SSN6 TUP1*) (*WT*) and its derivatives containing the *ssn6Δ9* allele or the *Δtup1::LEU2* allele. Units of β-galactosidase activity measured in cells growing in YEPD are given on the right. pLG312, pLGΔ312(Bgl). (B) The middle fragment in combination with NRE^{DIT} promotes a high level of expression in the absence of Ssn6 or Tup1. Wild-type, *ssn6*, and *tup1* cells containing pLGΔ312SΔSS(Bgl) with the indicated inserts at the *Bgl*II site were analyzed as described for panel A. pLGΔSS, pLGΔ312SΔSS(Bgl). (C and D) Analysis of expression of *DIT1-lacZ* translational fusion genes. Wild-type, *ssn6*, and *tup1* cells containing p(−537)DIT1-lacZ, p(−463)DIT1-lacZ, p(−114)DIT1-lacZ, and pNRE(−114)DIT1-lacZ were analyzed as described for panel A. (E) The chromosomal *DIT1* and *DIT2* genes are expressed in *ssn6* and *tup1* cells growing vegetatively. Northern blots were prepared with RNA purified from EG123 (wild-type) (lane 1), *Δtup1::LEU2* (lane 2), and *ssn6Δ9* (lane 3) cells growing in YEPD. As hybridization controls, the blots also contain RNA from the diploid strain LP112 (*MAT***a**/*MAT*α *SSN6*/*SSN6 TUP1*/*TUP1*) harvested during vegetative growth in YEPA (veg) (lane 4) and 12 h after transfer to sporulation medium (spo) (lane 5). One filter was hybridized with the radioactively labeled probes for *DIT1*, *DIT2*, and *PYK1* (*PYK1* not shown). A duplicate filter was hybridized with radioactively labeled probes for *HOP1*, *SPS4*, and *PYK1*.

noted only a minor increase in the expression of a *CYC1-lacZ* reporter gene containing NRE^{DIT} in the absence of Ssn6 or Tup1 (Fig. 5B, lines 1 and 2). In contrast, NRE^{DIT} plus the middle fragment, a combination which did not support expression of the *CYC1-lacZ* reporter gene in the wild-type EG123 strain (Fig. 5B, line 3) or in our standard diploid strain (Fig. 3B, line 5), led to a high level of β-galactosidase activity in the *ssn6* and *tup1* strains (Fig. 5B, line 3). These results indicate that in the absence of the Ssn6-Tup1 repression complex, NRE^{DIT} and a putative element in the middle fragment act synergistically to promote a high level of expression in vegetative cells. We note that whereas we had previously observed that the middle fragment had UAS activity in our standard diploid strain (Fig. 3B, line 4), this fragment gave little or no UAS activity in the EG123-derived strains (Fig. 5B, compare lines 4 and 1); the inability to detect UAS activity for this fragment in these strains may be due to the high basal level of β-galactosidase activity obtained with the *CYC1-lacZ* reporter gene.

We also monitored expression of the *DIT1-lacZ* translational fusion genes in *ssn6* and *tup1* strains. As anticipated, the *(−537)DIT1-lacZ* fusion gene, which contains NRE^{DIT}, was not expressed in wild-type EG123 cells during vegetative growth (Fig. 5C, line 1) but was expressed in the absence of Ssn6 or Tup1, with β-galactosidase activity being fivefold higher in cells lacking Tup1 than in cells lacking Ssn6. The *(−463)DIT1-lacZ* translational fusion gene, which lacks NRE^{DIT} yet gave a moderate level of sporulation-induced β-galactosidase activity in our standard diploid strain (Fig. 4B and C), was expressed at a higher level in the *tup1* strain than in the wild-type and *ssn6* strains (Fig. 5C, line 2). These results suggest that repression of *DIT1* gene expression during vegetative growth is mediated mostly by Ssn6-Tup1 acting through NRE^{DIT}. The observation that Tup1-dependent repression, however, also acted on the sequence downstream of NRE^{DIT} (Fig. 5C, line 2) might account for the low level of induction seen during sporulation with a *DIT1-lacZ* reporter gene lacking NRE^{DIT} (Fig. 4B and C).

The *(−114)DIT1-lacZ* translational fusion gene was expressed at approximately the same level in wild-type EG123, *ssn6*, and *tup1* cells (Fig. 5D, line 1). Insertion of NRE^{DIT} into this gene reduced expression in an Ssn6- and Tup1-dependent manner (Fig. 5D, line 2). However, in contrast to the combination of NRE^{DIT} and the middle fragment (Fig. 5B, line 3), NRE^{DIT} and a putative element in the start-site-containing fragment did not act synergistically in the absence of Ssn6 or Tup1 to promote increased expression in vegetative cells.

To determine whether Ssn6 and Tup1 were required for repression of the chromosomal *DIT1* and *DIT2* genes during vegetative growth, RNAs isolated from isogenic wild-type, *ssn6*, and *tup1* strains were analyzed by Northern blotting. Both *DIT1* and *DIT2* transcripts were readily detected in RNAs from *ssn6* and *tup1* cells growing vegetatively but not in RNA from wild-type cells growing vegetatively (Fig. 5E, lanes 1 to 3). As a control, we also monitored expression of these genes in our standard diploid strain during vegetative growth and during sporulation (Fig. 5E, lanes 4 and 5); as expected, *DIT1* and *DIT2* transcripts were not present in RNA from *MAT***a**/*MAT*α cells growing vegetatively but were present in RNA from sporulating cells. To assess the specificity of Ssn6-Tup1-dependent repression of the *DIT* genes, we probed for transcripts from the early meiotic gene *HOP1* (24) and the middle sporu-

lation gene *SPS4* (22). Transcripts for these genes were not detected in vegetatively growing *ssn6* or *tup1* cells (Fig. 5E). In summary, these experiments indicate that repression of the *DIT1* and *DIT2* genes during vegetative growth requires Ssn6 and Tup1 and that the effect of the Ssn6-Tup1 repression complex is mediated through NRE^{DIT}.

DISCUSSION

The *DIT1* and *DIT2* genes, which are required for spore wall formation in *S. cerevisiae*, are expressed during the mid-late period of sporulation, with maximal transcript accumulation occurring at the time of prospore enclosure (7). In this study, we have identified an NRE, termed NRE^{DIT}, that is located between the start sites of transcription of these divergently transcribed genes and that prevents their expression during vegetative growth. NRE^{DIT}-mediated repression requires the well-characterized Ssn6-Tup1 repression complex. Repression through NRE^{DIT} is also effective on a heterologous reporter gene; in this case, however, repression is not relieved during sporulation. Derepression of *DIT1* during sporulation requires at least two *cis*-acting elements. One of these elements is located between the NRE and the TATA box (nt −463 to −114; middle fragment), and one is located within a fragment containing the TATA box and extending to the initiator ATG of the *DIT1* gene (nt −76 to +53; start-site-containing fragment). Additionally, a region of NRE^{DIT} which contains a region with sequence similarity to UAS^{SPS4} is required for full activation of *DIT1* during sporulation. Thus, NRE^{DIT} plays a dual role in regulation of *DIT1*: it mediates repression during vegetative growth, and it contributes to activation during sporulation.

A model for sporulation-specific expression of *DIT1*. We present the following model to explain our finding that three regions of the *DIT1* promoter are required for derepression during sporulation (Fig. 6). In this model, we propose that interactions among four regulatory factors control expression from the *DIT1* promoter. NRE^{DIT} is bound by two factors: sporulation activation factor (SAF), which binds to the UAS^{SPS4}-like element, and NRE repression factor (NRF), which binds downstream of SAF. Two additional regulatory factors bind downstream of NRE^{DIT}: binding factor 2 (BF2) binds to a site within the middle fragment, and BF3 binds to a site within the start-site-containing fragment. We note that the regulatory roles attributed to each factor could depend on several proteins, with each one interacting with a distinct site.

In vegetative cells, binding of NRF to NRE^{DIT} directs repression of *DIT1* (Fig. 6A). This repression requires that Ssn6 and Tup1 interact with NRF or indirectly control the activity of NRF. BF2 may also make a modest Tup1-dependent contribution to repression in these cells. For *ssn6* or *tup1* cells growing vegetatively, we propose that the absence of the Ssn6-Tup1 repression complex allows an interaction between NRF and BF2. This interaction leads to a form of BF2 that activates transcription at the *DIT1* promoter. We refer to this form as BF2* and depict it as a conformational isomer in which an activation domain is unmasked (Fig. 6B). In the absence of NRE^{DIT}, an interaction between BF2 and BF3 can partially substitute for the interaction between BF2 and NRF in promoting the change of BF2 to BF2*.

During the early part of the sporulation program in wild-type cells, the Ssn6-Tup1 repression complex remains bound to NRF and represses *DIT1*. We propose that midway through sporulation, a sporulation-specific event occurs (such as recruitment of a sporulation-specific protein or a phosphorylation event) that displaces Ssn6-Tup1 from NRF. NRF and BF2 are then free to interact; this leads to BF2*-dependent activa-

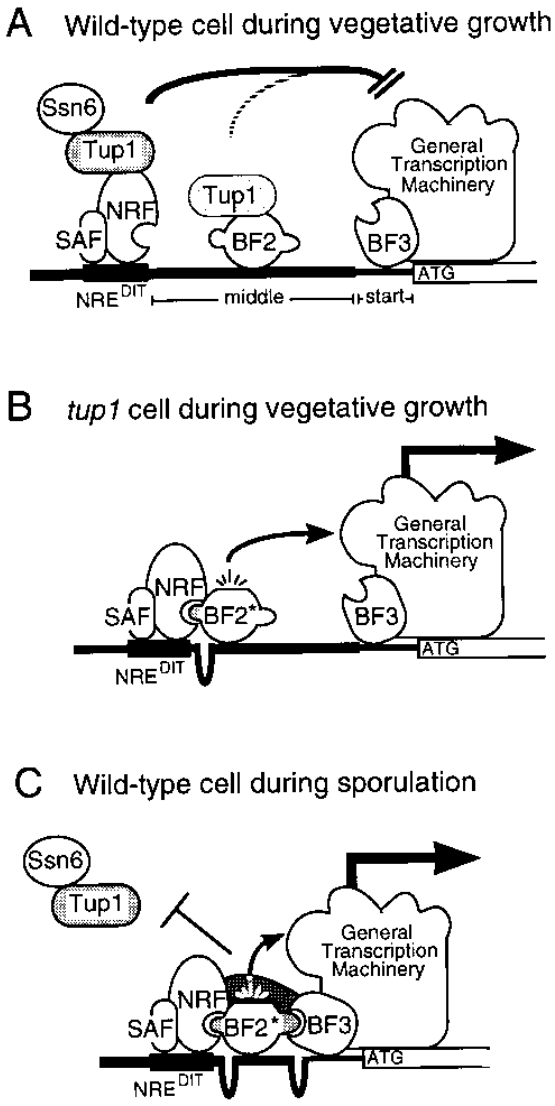

FIG. 6. Model for sporulation-specific expression of *DIT1*. We have identified three regions of DNA that are required for derepression of *DIT1* during sporulation. We propose that a regulatory protein or proteins binds to a site within each region: SAF binds to the UAS^{SPS4}-like element in NRE^{DIT} (nt −525 to −509), NRF binds downstream of the UAS^{SPS4}-like element within NRE^{DIT} (nt −505 to −462), BF2 binds to a site within the middle fragment (nt −463 to −114), and BF3 binds to a site within the start-site-containing fragment (nt −76 to +53). The three regions of DNA are denoted NRE^{DIT}, middle, and start in panel A. (A) NRE^{DIT} mediates Ssn6-Tup1-dependent repression of transcription in vegetative cells. During vegetative growth, NRF binds to NRE^{DIT} and recruits Ssn6-Tup1, which acts as a general repressor of transcription. BF2 may make a minor contribution to repression by interacting with Tup1. (B) In vegetative cells, the SAF-NRF complex bound at NRE^{DIT} interacts with BF2 to activate transcription in the absence of Ssn6-Tup1. Formation of the SAF-NRF · BF2 complex results in a form of BF2, denoted BF2*, that can activate transcription. In the absence of NRE^{DIT}, an interaction between BF2 and BF3 may partially substitute for the interaction between BF2 and SAF-NRF in promoting activation. (C) Sporulation-specific expression. A sporulation-specific event, shown here as recruitment of a sporulation-specific protein (dark shading), displaces Ssn6-Tup1 from NRF. The requirement for both BF2 and BF3 in derepression is depicted by the formation of a SAF-NRF · BF2 · BF3 complex. We suggest that the interactions between SAF-NRF and BF2 and between BF2 and BF3 are prerequisites for relief from Ssn6-Tup1-mediated repression. At the same time, the SAF-NRF · BF2 · BF3 complex promotes BF2*-dependent activation of transcription.

tion of transcription. We have depicted the consequence of the sporulation-specific event required for expression of *DIT1* during sporulation as formation of an NRF · BF2 · BF3 complex (Fig. 6C). This complex, which is resistant to Ssn6-Tup1, promotes formation of BF2*. Both an NRF · BF2 interaction and a BF2 · BF3 interaction may contribute to this change of BF2 to BF2*. We wish to emphasize that whereas BF3 is not required for expression from an NRE^{DIT}-containing promoter in *ssn6* or *tup1* cells during vegetative growth, BF3, as well as BF2, is essential for expression in wild-type cells during sporulation. We postulate that BF3 is required during sporulation because it plays an essential role in the sporulation-specific event that displaces Ssn6-Tup1 from NRF. If Ssn6-Tup1 is removed from vegetative cells by mutation, BF3 is no longer required. Furthermore, in cells lacking Ssn6 or Tup1, NRF and BF2 act synergistically to promote transcription whereas NRF and BF3 do not. This supports the idea that BF2 is needed for activation, whereas the role of BF3 is limited to derepression.

Complex interactions regulate sporulation-specific expression of *DIT1*. The observation that repression of a heterologous reporter gene containing NRE^{DIT} required Ssn6-Tup1 and that this repression was not relieved during sporulation suggested that the Ssn6-Tup1 repression complex retains activity during sporulation. Indeed, we found that repression of a *CYC1*(*UAS*)-*lacZ* reporter gene containing the α2 operator (25) was maintained in sporulating cells (data not shown). Our findings suggest that derepression requires complex interactions. Although the middle fragment and the start-site-containing fragment of *DIT1* each promoted constitutive expression of a reporter gene in our standard laboratory yeast strain, neither the combination of NRE^{DIT} and the middle fragment nor the combination of NRE^{DIT} and the start-site-containing fragment supported expression of a reporter gene during sporulation (Fig. 3B and Fig. 4A). We therefore conclude that both BF2 and BF3 are required to overcome NRF-mediated repression of *DIT1* during sporulation.

We found that NRE^{DIT} also makes a contribution to activation of *DIT1* (Fig. 4). The contribution of NRE^{DIT} to BF2-dependent activation of *DIT1* was evident in vegetative cells of strain EG123 that lacked either Ssn6 or Tup1 (Fig. 5B). We found that the *DIT1* middle fragment had barely detectable activity in promoting expression of a *CYC1-lacZ* reporter gene in wild-type cells of the EG123 strain as well as in the isogenic *tup1* and *ssn6* strains. In contrast, NRE^{DIT} and the middle fragment together promoted a high level of expression of the reporter gene during vegetative growth in *ssn6* and *tup1* cells but not in *SSN6 TUP1* cells (Fig. 5B). This experiment not only revealed that NRE^{DIT} could contribute to activation in vegetative cells in the absence of Ssn6-Tup1 but also indicated that under these conditions the start-site-containing fragment is not required for expression of the reporter gene.

Bipartite nature of NRE^{DIT}. By monitoring expression of *DIT1* and *DIT2* genes that contained sequential deletions into the 5′-flanking region, we initially mapped the NRE between nt −537 and −485 (Fig. 2 and data not shown). However, a 53-bp fragment that contained this region (nt −537 to −485) did not affect expression of the *CYC1*(*UAS*)-*lacZ* reporter gene (Fig. 3A). The observation that NRE^{DIT}, as defined in a heterologous reporter gene, extended beyond a boundary defined by deletion analysis could indicate functional redundancy of sequence elements within the *DIT1-DIT2* intergenic region. We did find, however, that a 44-bp fragment from NRE^{DIT} (nt 505 to −462), referred to as the minimal NRE, retained significant negative regulatory activity. Whereas the 76-bp NRE^{DIT} led to a 1,000-fold reduction in expression of the *CYC1*(*UAS*)-*lacZ* reporter gene, the 44-bp minimal NRE led to a 40-fold reduction in expression (Fig. 3A). We note that a study by Kihara et al. (26) showed that *SGA1*, which is expressed during the latter portion of the sporulation program, also contains an NRE which is required for repression during vegetative growth. We have detected no sequence similarity between the region containing the *SGA1* negative regulatory element and NRE^{DIT}.

We found that the sequence adjacent to the minimal NRE not only contributed to maximal repression in vegetative cells but also contributed to full activation of *DIT1* during sporulation. The fusion gene (*−505*)*DIT1-lacZ*, which contains the minimal 44-bp NRE, gave severalfold less β-galactosidase activity during sporulation than did (*−537*)*DIT1-lacZ*, which contains NRE^{DIT} (Fig. 4B and data not shown). Hepworth et al. (22) noted that *DIT1* has a 15-of-17-bp match with a sequence spanning UAS^{SPS4}, a short element that activates expression of *SPS4* midway through sporulation. This match maps within NRE^{DIT}, upstream of the minimal NRE sequence, coincident with the sequence that makes a modest contribution to both repression and activation of *DIT1*. Interestingly, although *SPS4* does not appear to be subject to negative control during vegetative growth, a factor that is present in extracts of vegetative cells binds to UAS^{SPS4} (22). We have found that this activity, which we refer to as SAF (Fig. 6), also binds to the UAS^{SPS4}-like sequence in NRE^{DIT} (data not shown). Hepworth et al. (22) proposed that a sporulation-specific event converts SAF to an activator during sporulation. Thus, SAF might contribute to repression of *DIT1* in vegetative cells by interacting with NRF and might also promote activation of both *SPS4* and *DIT1* during sporulation. At this time, we can not distinguish between the contributions of SAF and NRF to formation of the putative NRF · BF2 · BF3 complex. Finally, we note that we found that a 29-bp fragment spanning the UAS^{SPS4}-like element in NRE^{DIT} had neither NRE nor UAS activity in our assays (data not shown and Fig. 3B). Thus, in contrast to the UAS^{SPS4} element from the *SPS4* gene, the UAS^{SPS4}-like element from NRE^{DIT} cannot activate on its own.

We have proposed that a sporulation-specific event such as binding of a sporulation-specific protein or a phosphorylation event is required to displace Ssn6-Tup1 from NRE^{DIT}, leading to activation of *DIT1*. The idea that a sporulation-specific event renders the NRF · BF2 · BF3 complex resistant to Ssn6-Tup1 can be compared with the proposal that differential phosphorylation of Mig1 is responsible for regulating the ability of this protein to mediate repression (32, 48, 51). Expression of early meiotic genes, which requires that the URS1-binding protein Ume6 be converted from a repressor to an activator by recruiting Ime1, provides an example of a sporulation-specific event that inhibits repression and promotes activation (4, 5, 40, 45).

Redundant negative control. We found that the *DIT1* sequence from nt −463 to +53, which does not contain NRE^{DIT} but contains the middle fragment and the start-site-containing fragment, supported a 12-fold induction of reporter gene expression during sporulation (Fig. 4B). This suggested that some sporulation-specific regulation is imparted to the *DIT1* gene in the absence of NRE^{DIT}. At this time, we cannot distinguish between redundant negative control and the possibility that a component of the activation system is sporulation specific. We cannot readily explain why expression of a *DIT1* gene containing 463 bp of 5′-flanking sequence gave comparable levels of expression during vegetative growth and sporulation when assessed by Northern blot analysis (Fig. 1). The finding that the reporter gene, (*−463*)*DIT1-lacZ*, was more active in *tup1* cells than in wild-type cells during vegetative

growth suggests, however, that there may be a component of repression that is independent of NRE^{DIT} (Fig. 5C). Repression by Tup1 in the absence of Ssn6 has been noted previously (49). The notion of two Tup1-dependent repression sites acting on *DIT1* is reminiscent of Ssn6-Tup1-mediated repression of *SUC2*. Genetic evidence suggests that repression of *SUC2* by Ssn6-Tup1 is mediated by another factor(s) in addition to Mig1 (51).

Possible role for BF3 in mediating NRF · BF2-activated transcription in sporulating cells. As discussed above, our model proposes that in sporulating cells a putative sporulation-specific event promotes the formation of an NRF · BF2 · BF3 complex that is resistant to repression by Ssn6-Tup1. Our data indicate that the start-site-containing fragment is essential for derepression; we have suggested that BF3 binds to this fragment and serves a role in the events that lead to derepression. The observation that the start-site-containing fragment supports a high level of basal transcription raises the possibility that BF3, in addition to contributing to derepression, could serve as a transcriptional activator. We note that Kuo and Greyhack (27) identified an Mcm1-binding site just downstream of the start site of transcription of *DIT1* (nt +13 to +23). Mcm1 has been shown to have weak activation activity (16). We have found, however, that mutation of this Mcm1-binding site in p(−537)DIT1 does not appear to affect *DIT1* expression (data not shown). It is also possible that BF3 does not bind to DNA directly but that it is a component of the RNA polymerase II holoenzyme. In this case, the BF3-containing enzyme might be dedicated to the transcription of a subset of sporulation-specific genes.

Our finding that sporulation-specific expression of *DIT1* requires an element at or downstream of the TATA box has precedents among other sporulation-specific genes. For example, UAS_H and URS1 have been identified as upstream regulatory elements controlling meiotic expression of *HOP1*. A 100-bp DNA fragment containing these two sites does not support sporulation-specific expression of a reporter gene, whereas a 225-bp fragment that contains these sites and extends 18 bp past the initiator ATG codon of *HOP1* does support sporulation-specific expression (52).

In summary, we have identified a potent NRE, NRE^{DIT}, that prevents expression of the mid-late sporulation-specific genes, *DIT1* and *DIT2*, during vegetative growth. It will be interesting to determine whether this element regulates expression of other sporulation-specific genes and whether it also has a role in regulating expression of non-sporulation-specific genes. Our preliminary characterization of the *cis*-acting elements in the *DIT1* promoter suggests that interactions among at least four regulatory factors are required for maximum levels of derepression of *DIT1* during sporulation. Future studies aimed at delimiting regulatory sites and identifying regulatory proteins will shed light on the mechanisms controlling expression of *DIT1*.

ACKNOWLEDGMENTS

We thank Peter Briza for construction of the pPB series of plasmids and the initial series of plasmids containing deletions of *DIT* sequence. We thank Peter Briza and Michael Breitenbach for helpful discussions during the course of this study and Robert Trumbly and Alexander Johnson for providing strains. We also thank Jack Greenblatt, Julia Pak, and Jason Tanny for valuable comments regarding the manuscript.

S.R.H. was supported by a studentship from the Natural Sciences and Engineering Research Council (Canada). This work was supported by a Medical Research Council (Canada) grant (MA-6826) to J.S.

REFERENCES

1. **Anderson, S. F., C. M. Steber, R. E. Esposito, and J. E. Coleman.** 1995. UME6, a negative regulator of meiosis in *Saccharomyces cerevisiae*, contains a C-terminal Zn_2Cys_6 binuclear cluster that binds the URS1 DNA sequence in a zinc-dependent manner. Protein Sci. **4:**1832–1843.
2. **Balasubramanian, B., C. V. Lowry, and R. S. Zitomer.** 1993. The Rox1 repressor of the *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. **13:**6071–6078.
3. **Barral, Y., S. Jentsch, and C. Mann.** 1995. G_1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. Genes Dev. **9:**399–409.
4. **Bowdish, K. S., and A. P. Mitchell.** 1993. Bipartite structure of an early meiotic upstream activation sequence from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**2172–2181.
5. **Bowdish, K. S., H. E. Yuan, and A. P. Mitchell.** 1995. Positive control of yeast meiotic genes by the negative regulator UME6. Mol. Cell. Biol. **15:**2955–2961.
6. **Briza, P., M. Eckerstorfer, and M. Breitenbach.** 1994. The sporulation-specific enzymes encoded by the *DIT1* and *DIT2* genes catalyze a two-step reaction leading to a soluble LL-dityrosine-containing precursor of the yeast spore wall. Proc. Natl. Acad. Sci. USA **91:**4524–4528.
7. **Briza, P., M. Breitenbach, A. Ellinger, and J. Segall.** 1990. Isolation of two developmentally regulated genes involved in spore wall maturation in *Saccharomyces cerevisiae*. Genes Dev. **4:**1775–1789.
8. **Buckingham, L. E., H.-T. Wang, R. T. Elder, R. M. McCarroll, M. R. Slater, and R. E. Esposito.** 1990. Nucleotide sequence and promoter analysis of *SPO13*, a meiosis-specific gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78:**9406–9410.
9. **Byers, B.** 1981. Cytology of the yeast life cycle, p. 59–96. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein.** 1984. A suppressor of *SNF1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107:**19–32.
11. **Clancy, M., J. B. Buten-Magee, D. J. Straight, A. L. Kennedy, R. M. Partridge, and P. T. Magee.** 1983. Isolation of genes expressed preferentially during sporulation in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80:**3000–3004.
12. **Coe, J. G. S., L. E. Murray, and I. W. Dawes.** 1994. Identification of a sporulation-specific promoter regulating divergent transcription of two novel sporulation genes in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **244:**661–672.
13. **Cooper, J. P., S. Y. Roth, and R. T. Simpson.** 1994. The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **8:**1400–1410.
14. **Deckert, J., A. M. Rodriguez Torres, J. T. Simon, and R. S. Zitomer.** 1995. Mutational analysis of ROX1, a DNA-bending repressor of hypoxic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**6109–6117.
15. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
16. **Elble, R., and B.-K. Tye.** 1991. Both activation and repression of a-mating-type-specific genes in yeast require transcription factor MCM1. Proc. Natl. Acad. Sci. USA **88:**10866–10970.
17. **Elledge, S. J., Z. Zhou, J. B. Allen, and T. A. Navas.** 1993. DNA damage and cell cycle regulation of ribonucleotide reductase. Bioessays **15:**333–339.
18. **Esposito, R. E., and S. Klapholz.** 1981. Meiosis and ascospore development, p. 211–287. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Gailus-Durner, V., J. Xie, C. Chintamaneni, and A. K. Vershon.** 1996. Participation of the yeast activator Abf1 in meiosis-specific expression of the *HOP1* gene. Mol. Cell. Biol. **16:**2777–2786.
20. **Guarente, L., and T. Mason.** 1983. Heme regulates transcription of the *CYC1* gene of *S. cerevisiae* via an upstream activation site. Cell **32:**1279–1286.
21. **Guarente, L., and E. Hoar.** 1984. Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when placed downstream of the "TATA box." Proc. Natl. Acad. Sci. USA **81:**7860–7864.
22. **Hepworth, S. R., L. K. Ebisuzaki, and J. Segall.** 1995. A 15-base-pair element activates the *SPS4* gene midway through sporulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**3934–3944.
23. **Herschbach, B. M., M. B. Arnaud, and A. D. Johnson.** 1994. Transcriptional repression directed by the yeast α2 protein *in vitro*. Nature **370:**309–311.
24. **Hollingsworth, N. M., L. Goetsch, and B. Byers.** 1990. The *HOP1* gene encodes a meiosis-specific component of yeast chromosomes. Cell **61:**73–84.
25. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. SSN6-TUP1 is a general repressor of transcription in yeast. Cell **68:**709–719.
26. **Kihara, K., M. Nakamura, R. Akada, and I. Yamashita.** 1991. Positive and negative elements upstream of the meiosis-specific glucoamylase gene in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **226:**383–392.
27. **Kuo, M.-H., and E. Grayhack.** 1994. A library of yeast genomic Mcm1

binding sites contains genes involved in cell cycle control, cell wall and membrane structure, and metabolism. Mol. Cell. Biol. **14:**348–359.
28. **Luche, R. M., R. Sumrada, and T. G. Cooper.** 1990. A *cis*-acting element present in multiple genes serves as a repressor protein binding site for the yeast *CAR1* gene. Mol. Cell. Biol. **10:**3884–3895.
29. **Matallana, E., L. Franco, and J. E. Perez-Ortin.** 1992. Chromatin structure of the yeast *SUC2* promoter in regulatory mutants. Mol. Gen. Genet. **231:** 395–400.
30. **Mitchell, A. P.** 1994. Control of meiotic gene expression in *Saccharomyces cerevisiae*. Microbiol. Rev. **58:**56–70.
31. **Mukai, Y., S. Harashima, and Y. Oshima.** 1991. AAR1/TUP1 protein, with a structure similar to that of the β subunit of G proteins, is required for **a**1-α2 and α2 repression in cell type control of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**3773–3779.
32. **Östling, J., M. Carlberg, and H. Ronne.** 1996. Functional domains in the Mig1 repressor. Mol. Cell. Biol. **16:**753–761.
33. **Ozsarac, N., M. Bhattacharyya, I. W. Dawes, and M. J. Clancy.** 1995. The *SPR3* gene encodes a sporulation-specific homologue of the yeast CDC3/10/11/12 family of bud neck microfilaments and is regulated by Abf1. Gene **164:**157–162.
34. **Park, H.-D., R. M. Luche, and T. G. Cooper.** 1992. The yeast Ume6 gene product is required for transcriptional repression mediated by the *CAR1* URS1 repressor binding site. Nucleic Acids Res. **20:**1909–1915.
35. **Percival-Smith, A., and J. Segall.** 1984. Isolation of DNA sequences preferentially expressed during sporulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**142–150.
36. **Primerano, D.** Personal communication.
37. **Prinz, S., F. Klein, H. Auer, D. Schweizer, and M. Primig.** 1995. A DNA binding factor (UBF) interacts with a positive regulatory element in the promoters of genes expressed during meiosis and vegetative growth in yeast. Nucleic Acids Res. **23:**3449–3456.
38. **Ronne, H.** 1995. Glucose repression in fungi. Trends Genet. **11:**12–17.
39. **Roth, S. Y.** 1995. Chromatin-mediated transcriptional repression in yeast. Curr. Opin. Genet. Dev. **5:**168–173.
40. **Rubin-Bejerano, I., S. Mandel, K. Robzyk, and Y. Kassir.** 1996. Induction of meiosis in *Saccharomyces cerevisiae* depends on conversion of the transcriptional repressor Ume6 to a positive regulator by its regulated association with the transcriptional activator Ime1. Mol. Cell. Biol. **16:**2518–2526.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Schultz, J., L. Marshall-Carlson, and M. Carlson.** 1990. The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**4744–4756.
43. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
44. **Smith, R. L., M. J. Redd, and A. D. Johnson.** 1995. The tetratricopeptide repeats of Ssn6 interact with the homeo domain of α2. Genes Dev. **9:**2903–2910.
45. **Steber, C. M., and R. E. Esposito.** 1995. *UME6* is a central component of a developmental regulatory switch controlling meiosis-specific gene expression. Proc. Natl. Acad. Sci. USA **92:**12490–12494.
46. **Strich, R., R. T. Surosky, C. Steber, E. Dubois, F. Messenguy, and R. E. Esposito.** 1994. *UME6* is a key regulator of nitrogen repression and meiotic development. Genes. Dev. **8:**796–810.
47. **Struhl, K.** 1995. Yeast transcriptional regulatory mechanisms. Annu. Rev. Genet. **29:**651–674.
48. **Treitel, M. A., and M. Carlson.** 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92:**3132–3136.
49. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature **369:**758–761.
50. **Tzamarias, D., and K. Struhl.** 1995. Distinct TPR motifs of CYC8 are involved in recruiting the CYC8-TUP1 corepressor complex to differentially regulated promoters. Genes Dev. **9:**821–831.
51. **Vallier, L. G., and M. Carlson.** 1994. Synergistic release from glucose repression by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. Genetics **137:**49–54.
52. **Vershon, A. K., N. M. Hollingsworth, and A. D. Johnson.** 1992. Meiotic induction of the yeast *HOP1* gene is controlled by positive and negative regulatory sites. Mol. Cell. Biol. **12:**3706–3714.
53. **Wickner, R. B.** 1974. Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5′-monophosphate into deoxyribonucleic acid in vivo. J. Bacteriol. **117:**252–260.
54. **Williams, F. E., U. Varanasi, and R. J. Trumbly.** 1991. The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cell. Biol. **11:**3307–3316.
55. **Zhang, L., and L. Guarente.** 1994. Evidence that TUP1/SSN6 has a positive effect on the activity of the yeast activator HAP1. Genetics **136:**813–817.
56. **Zhou, Z., and S. J. Elledge.** 1992. Isolation of *crt* mutants constitutive for transcription of the DNA damage inducible gene *RNR3* in Saccharomyces cerevisiae. Genetics **131:**851–866.
57. **Zitomer, R. S., and C. V. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1–11.