CHARACTERIZATION OF THE PROMOTER AND UPSTREAM ACTIVATION SEQUENCES OF SPORULATION-SPECIFIC GENES *SPR1* (*SSG1*) AND *SPR2* IN *SACCHAROMYCES CEREVISIAE*

DISSERTATION

Submitted to the Graduate College

of

Marshall University

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy

By

Sharmini S. Soosaithasan

Huntington,

West Virginia

1998

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ABSTRACT

Sporulation is a developmental process in which temporally distinct sets of genes regulate the processes of meiosis and ascospore formation. Sporulation-specific genes are classified into early, middle, and late genes based on their peak time of expression during sporulation. Regulation of two late genes, *SPR1* and *SPR2,* was studied. The function of *SPR2* is unknown. Its regulatory region contains two Mid Sporulation Elements (MSE) repeated in tandem that are sufficient to confer sporulation-specific regulation to a *CYC1-lacZ* reporter. These sequences have UAS (upstream activation sequence) properties, as each 14 base pair element is sufficient to confer sporulation-specific regulation independent of orientation. A greater than 5-fold higher induction is observed with the tandem MSEs compared to the individual elements. Mutations within a GC rich region indicate that these sequences are necessary for MSE function.

The *SPR1* gene encodes a 1,3-β-glucanase that is believed to be involved in spore wall maturation. The 5' regulatory region of *SPR1* was studied by constructing a series of translational and transcriptional fusions. Studies using the translational fusions suggest that sequences from -268 to +1 are sufficient to confer sporulation-specific regulation, and that *SPR1* is controlled by a TATA-less promoter that lies between –151 to +1. In addition, homology searches revealed two sequences with weak matches to known activation sequences: (a) sequences from –293 to –279 have 10/15 matches to the UAS of *SPS4*; (b) sequences from –252 to –236 have 12/17 matches to the MSE of *SPR2*. Internal deletion and transcriptional fusion

studies, taken together, indicate that both the UAS-like and MSE-like sequences are necessary but not sufficient for temporal regulation of *SPR1*. This evidence suggests that *SPR1* is regulated by a composite promoter with essential elements lying downstream of –268 and other weaker positive elements which are dispersed from –268 to –762.

Studies with mutants of *ime2, ume6* and *ndt80* suggest that all three proteins are positive regulators of the *SPR1* and *SPR2* genes. Distinct banding patterns observed during mobility shift assays with extracts from vegetative and sporulating cells, suggest that different protein complexes bind the regulatory sequences during vegetative growth and sporulation. Recombinant Ndt80p shows DNA protein interactions with both *SPR1* and *SPR2* probes. This evidence suggests that Ndt80p regulates *SPR2* via the MSE, while it regulates *SPR1* via other sequences.

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To the memory of my mother

Regina Manonmani Soosaithasan

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1.0 LITERATURE REVIEW

One of the fundamental decisions among eukaryotic cells is to exit mitosis and enter a differentiation pathway. In *Saccharomyces cerevisiae*, sporulation is a cellular differentiation pathway which is controlled by both genetic and environmental factors. The environmental signals, fermentable carbon or nitrogen deprivation, cause mitotic cell cycle arrest at the G1 phase. The genetic signal, expression of genes encoding the **a**/α repressor in heterozygous diploid cells, leads to entry into meiosis. Continued starvation ensures the completion of meiosis which results in ascospore formation and packaging of haploid ascospores in a sac-like structure called the ascus (Mitchell et al., 1990; Sia and Mitchell, 1995).

1.1a Model System

S. cerevisiae was used to study gene regulation because it is a relatively simple lower eukaryote that can be manipulated genetically and physiologically for experimental purposes; it also remains viable even under the most stringent nutritional conditions. In addition, yeast cells are good tools to isolate dominant and recessive mutations, to monitor timing and progression of events by interrupting meiosis, and to examine gene function by gene replacement procedures. This unicellular eukaryote is preferred over prokaryotes because it has characteristics similar to higher eukaryotes, such as posttranslational modifications, signal transduction systems and true mitotic and meiotic divisions. For example, at least six different pathways containing MAPKs (mitogen activated protein kinase) or their regulators have provided a wealth of information on how other eukaryotes respond to various extracellular signals via homologous MAPK pathways

(Choi et al., 1994; Johnson et al., 1994; Herskowitz et al., 1995).

1.1b *S. cerevisiae* Life Cycle

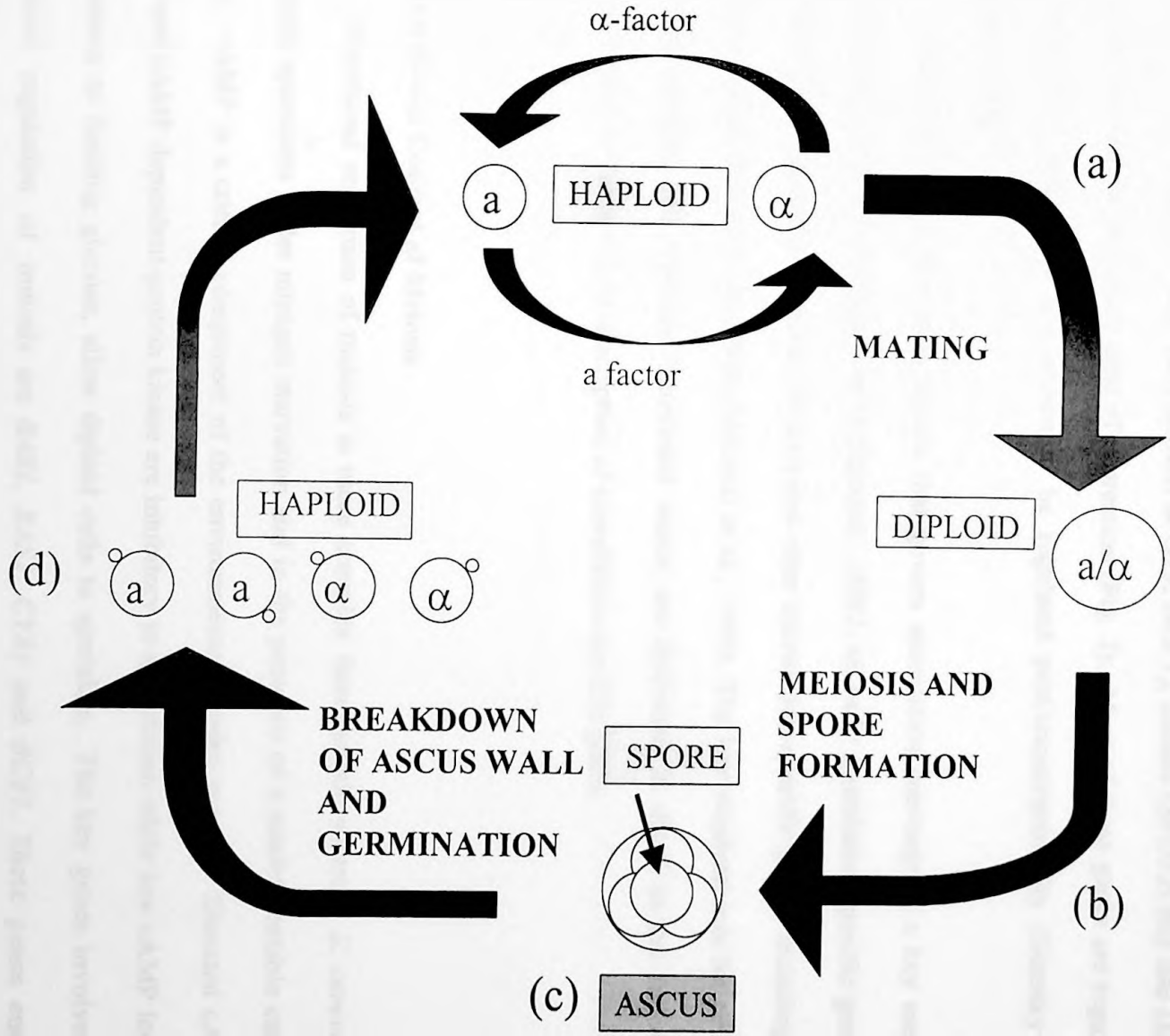
The life cycle of *S. cerevisiae* involves sexual and asexual reproduction. During asexual reproduction haploid and diploid cells divide via mitosis in rich or complete minimal medium; this is sometimes called vegetative growth. Cell division results in a single mother cell giving rise to a daughter cell smaller than the mother cell. Daughter cells must increase in size before initiating chromosomal replication and entering mitosis (Herskowitz et al., 1988). Sexual reproduction involves mating between haploid **a** and α cell types to give rise to a non-mating **a**/α cell (Fig. 1).

Cell type, an important determinant of cell fate, involves the expression of the alleles at the MAT (**ma**ting **t**ype) locus. Depending on the MAT allele present, haploid cells are either mating type MAT**a** or MATα, and the resulting diploid cells become heterozygous MAT**a**/ MATα (Herskowitz et al., 1992; Goutte et al., 1988). Diploids which are homozygous MAT**a**/ MAT**a** or MATα/ MATα are rarely found in nature, but can be made by mitotic crossing over for experimental purposes.

1.1c Major Events of Sporulation

Only MAT**a**/MATα diploid cells enter meiosis following starvation, whereas haploids simply arrest at G1 phase. Sporulation is a developmental process involving meiosis and ascospore formation. The major events of sporulation include premeiotic DNA synthesis,

Figure 1: *S. cerevisiae* Life Cycle. The *S. cerevisiae* life cycle consists of both haploid and diploid cells. (a) Haploid **a** and α cells mate to form the diploid heterozygous **a**/α cell. (b) The diploid cell is capable of entering sporulation upon nitrogen starvation. (c) At the end of sporulation four recombinant haploid spores are packaged in an ascus. (d) The cell wall breaks down releasing two **a** and two α spores.


α-factor
a
HAPLOID
α
(a)
MATING
a factor
DIPLOID
a/α
HAPLOID
(d)
a
a
α
α
BREAKDOWN
OF ASCUS WALL
AND
GERMINATION
SPORE
MEIOSIS AND
SPORE
FORMATION
(b)
(c)
ASCUS

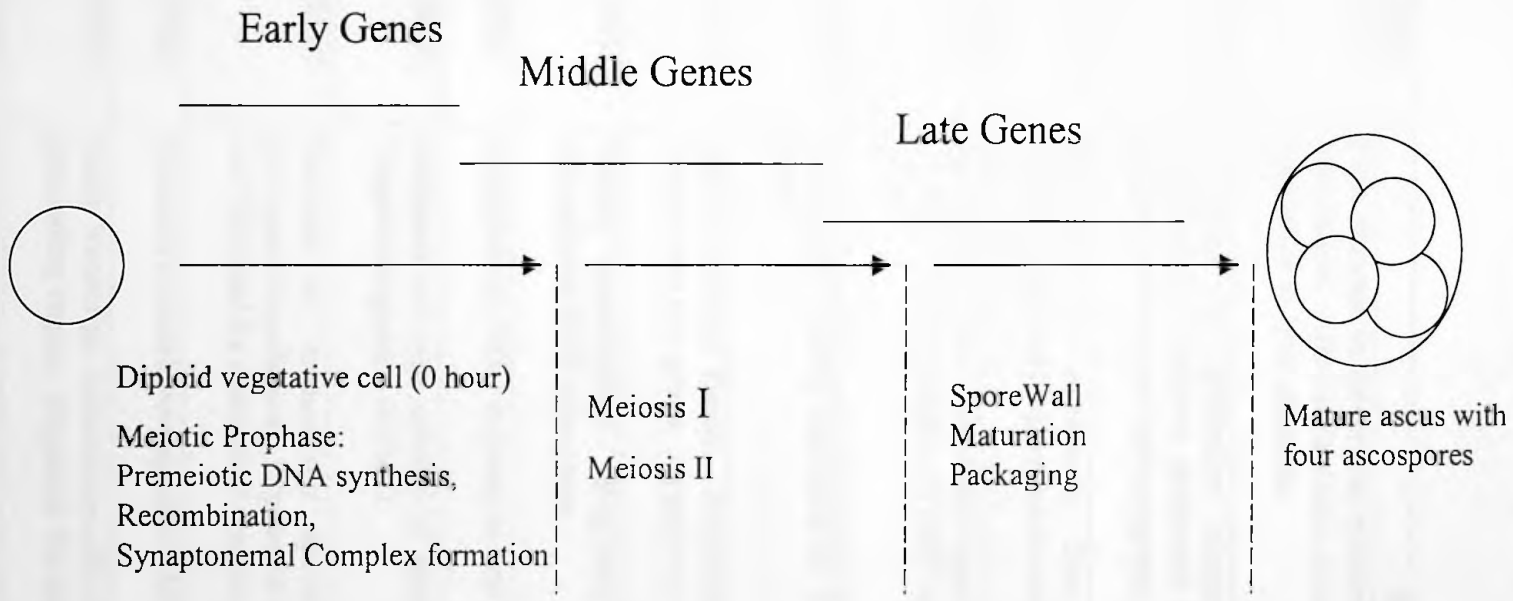
genetic recombination, formation of the synaptonemal complex, completion of meiosis I and meiosis II, and packaging of the four recombinant haploid spores in the ascus. All of these events are regulated by distinct sets of genes expressed in a temporal manner. All sporulation-specific genes are classified into early (*IME2*, *SPO13*, *HOP1*), middle (*SPO12*) and late (*SPR1*, *SPS2*) genes based on their peak time of expression (Fig. 2). Most of these genes are regulated transcriptionally and a few are known to be regulated post-transcriptionally (Surosky and Esposito, 1992).

Both genetic and starvation signals that govern sporulation converge at a key meiotic regulatory gene called *IME1* (inducer of meiosis). *IME1*, an early sporulation-specific gene, is also a positive regulator of meiosis. It activates other sporulation-specific genes, including the early gene *IME2* (Kassir et al., 1988; Mitchell et al., 1990). The key requirements for cells to enter into meiosis, cell type and nutritional status, are discussed in detail in the following sections. Refer to Table 1 for a description of sporulation-specific genes.

1.2 Nutritional Control of Meiosis

Nutritional regulation of meiosis is more complex than genetic control. *S. cerevisiae* efficiently sporulates under nitrogen starvation and in the presence of a nonfermentable carbon source. cAMP is a critical component of the environmental sensing system. Elevated cAMP levels and cAMP dependent-protein kinase are inhibitory to sporulation while low cAMP levels, in response to limiting glucose, allow diploid cells to sporulate. The key genes involved in nutritional regulation of meiosis are *RAS1, RAS2, CYR1* and *BCY1*. These genes encode membrane-associated GTP binding proteins Ras1p and Ras2p, adenylate cyclase and the

Figure 2: Temporal Expression of Sporulation-Specific Genes. Sporulation-specific genes are classified into groups based on their peak time of expression during sporulation. (a) Early genes govern prophase I which includes chromosomal pairing, recombination and synaptonemal complex formation. (b) Middle genes are involved in completion of meiosis I and II. (c) Late genes are involved in spore wall formation, packaging and maturation of ascospores. Time of expression varies depending on the strain under consideration.



Regulatory genes: *UME6*, *NDT80*
IME1, *IME2*

Target genes:	*IME1*, *IME2*	*SPO12*, meiosis I division	*SPR2*, (function unknown)
	DMC1, *HOP1*	*CLB*s, B type cyclins	*SPR1*, exo-1,3-β-glucanase activity
	ZIP1,		

Strain SK-1	7 hr	9 hr	12 hr
Strain GKY5	9 hr	11 hr	15 hr

Table 1: Functions of Key Sporulation-Specific Genes

Gene	Description
DIT1	First enzyme in dityrosine synthesis pathway. Transcribed during spore-wall maturation. Allows dityrosine accumulation in the outer spore wall and confers resistance to lytic enzymes.
DIT2	Required for dityrosine biosynthesis. Transcribed during spore-wall maturation. Allows dityrosine accumulation in the outer spore wall and confers resistance to lytic enzymes.
HOP1	Expressed early in meiosis. Zinc finger protein which localizes to the lateral elements and unsynapsed axial elements of the synaptonemal complex.
IDS2	Encodes an *IME2*-dependent signal protein that is required for down-regulation of *IME1*, autoregulation of *IME2* and expression of middle and late (not early) genes.
IME1	Early gene directly repressed by *RME1*. Required for transcription of meiotic genes.
IME2	Ser/Thr protein kinase. Positively regulated by *IME1* and stimulates early, middle and late genes. Negatively regulates *IME1*.
NDT80	Middle transcription. Regulates middle and other sporulation genes by recognizing MSE sequences.
RIM1	Required for *IME1* expression and proper time of appearance of mature spores.
RME1	Mediates cell-type control of sporulation and is directly repressed by a1/α2. Negative regulator of *IME1*.
SGA1	Encodes an intracellular sporulation-specific glucoamylase involved in glycogen degradation. Expressed late in sporulation and is regulated by *IME2* via NRE and by nutritional control via UAS site.
SMK1	Encodes a sporulation-specific MAP kinase.
SPO11	Early transcript; meiosis-specific regulation is dependent on URS1 site within the coding region. Required for chromosomal pairing.
SPR1	Late gene which encodes sporulation-specific 1,3-β-glucanase.

Table 1: Continued

SPR2	Encodes sporulation-specific late transcript. Function unknown.
SPS1	Ser/Thr protein kinase. Homologue of *STE20*. Expressed in mid/late meiosis and is required for spore wall formation.
SPS100	Induced late in sporulation. Required for cell wall maturation.
UME1-5	Regulators of early meiotic gene expression. Required for repression of early meiotic transcription and instability of early meiotic mRNA.
UME6	Encodes a C6 zinc finger URS1 binding protein, required for repression and induction of early meiotic genes. Requires Ime1p and Rim11p for meiosis specific transcription.

regulatory subunit of cAMP-dependent protein kinase, respectively (Fig. 3). *TPK1*, *TPK2* and *TPK3* encode the catalytic subunits of cAMP-dependent protein kinase (Tanaka et al., 1989). Ras1p and Ras2p are regulators of adenylate cyclase activity. The nutritional signal is communicated to the cell by the levels of cAMP which are governed by adenylate cyclase. cAMP binds to the regulatory subunit of cAMP-dependent protein kinase which then releases the catalytic subunits. The regulatory subunit inhibits the kinase in the absence of cAMP. Catalytic subunit mutants sporulate without starvation suggesting that at least part of the regulation of entry into meiosis is negatively regulated by phosphorylation. Although the phosphorylated target may regulate entry into meiosis by blocking *IME1* expression or by inactivating Ime1p, its identity is currently unknown.

Other components of this signal transduction pathway include *CDC25*, which encodes a positive regulator of Ras2p and *IRA1* (*PPD1*), which encodes a negative regulator of Ras2p. According to a model proposed by Tanaka et al. (1989), Ira1p may be involved in converting Ras2-GTP to Ras2-GDP, whereas Ras1p and Ras2p act as stimulators of adenylate cyclase. Thus, mutations in the *cyr1*, *cdc25*, *ras1* and *ras2* genes block activation of cAMP-dependent protein kinase and allow entry into sporulation in rich medium, whereas mutations in *bcy1*, *ira1* and a dominant negative $ras2^{val19}$ mutant cause high constitutive levels of cAMP-dependent protein phosphorylation and prevent sporulation even under starvation (Tanaka et al., 1989).

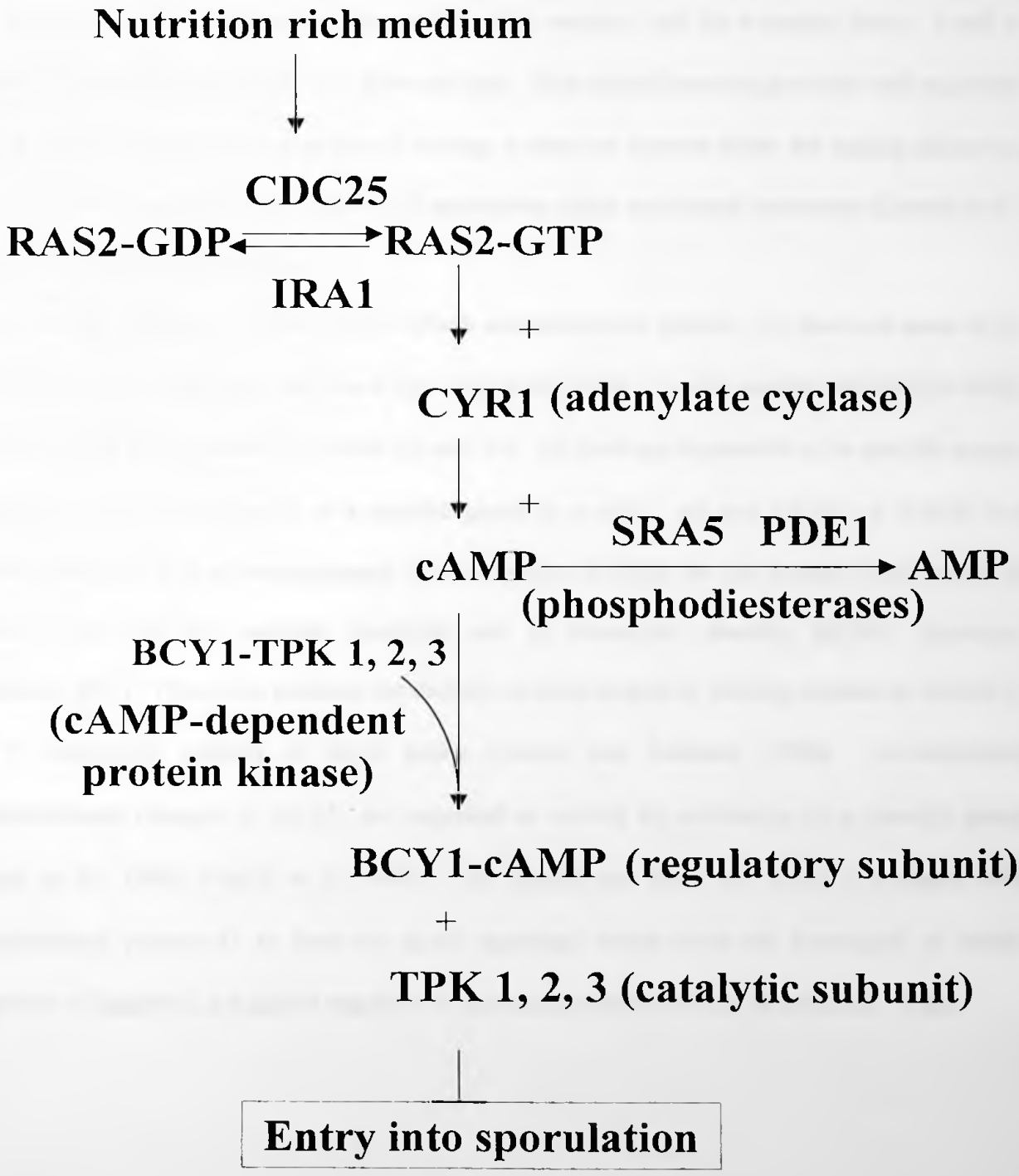
1.3 Genetic Control of Sporulation

1.3a Cell Type

Cell type is determined by alleles at the MAT locus (Rine and Herskowitz, 1987). Cells

Figure 3: Nutritional Control of Meiosis. cAMP is an important component of the environment sensing system. Elevated cAMP levels and adenylate cyclase activity are inhibitory to sporulation. Low cAMP levels in response to low levels of glucose allow cells to sporulate. *CDC25* is a positive regulator of *RAS2; IRA1* is a negative regulator of *RAS2; CYR1* encodes adenylate cyclase; *BCY1* encodes the regulatory unit of cAMP dependent protein kinase, and *TPK* 1,2,3 encode the catalytic subunits of cAMP-dependent protein kinase.

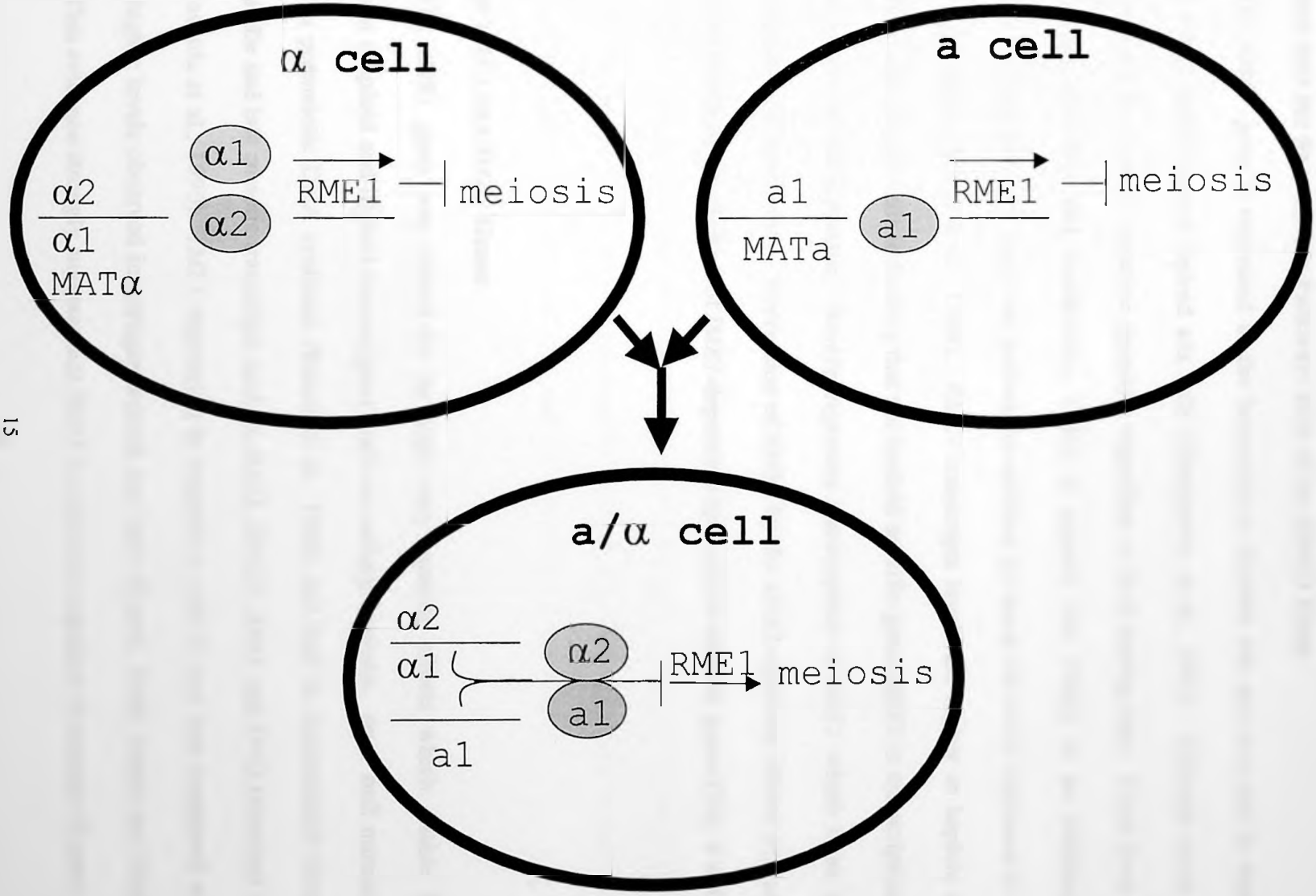
NUTRITIONAL CONTROL



become haploid **a**, α or diploid **a**/α by expressing either one or both alleles (Fig. 4). Haploid **a** or α cells produce their respective mating factors and cell surface receptors. For instance, an **a** cell will express the MAT**a** allele, the α cell surface receptor, and the **a** mating factor. **a** and α haploid cells mate and give rise to a third cell type. This diploid heterozygous **a**/α cell expresses both **a** and α alleles and is incapable of mating; it does not express either the mating factors or the cell surface receptor but is capable of sporulation under nutritional starvation (Goutte et al., 1988; Herskowitz et al., 1992).

a cells express the MAT**a** allele which encodes the a1 protein. a1 does not seem to be essential for the **a** cell type, but has a key role in **a**/α cells. α cells express the MATα allele which encodes homeodomain proteins α1 and α2. α1 turns on expression of α specific genes, while α2 turns off expression of **a** specific genes in α cells. α2 and MCM1, a MADS box protein, bind DNA as a heterotetramer. (The acronym, MADS, for the protein family name is derived from the first member identified and its phenotype, namely, MCM1, *Agamous*, *Deficiens*, SRF). These two products act directly on their targets by binding sequences located in the 5' regulatory regions of target genes (Goutte and Johnson, 1988). α1-dependent conformational changes of MCM1 are regarded as crucial for activation of α specific genes (Mead et al., 1996; Primig et al., 1991). In diploid **a**/α cells, α2 forms a complex with homeodomain protein a1 to form the a1/α2 repressor which turns off expression of *RME1* (regulator of meiosis), a negative regulator of sporulation (Mitchell and Herskowitz, 1986).

Figure 4: Determination of Cell Type. Cell type is determined by the mating type locus (MAT). Haploid cells express either MAT**a** or MATα and become **a** or α cells while heterozygous cells express both MAT**a** and MATα and become **a**/α cells. Haploid **a** and α cells do not enter meiosis as these cells produce *RME1* (repressor of meiosis). Diploid cells produce **a1**/α**2**, a repressor of *RME1* and enter meiosis when starved for nitrogen and carbon sources.


α cell
α1
α2
α2
α1
MATα
RME1
meiosis
a cell
a1
a1
MATa
RME1
meiosis
a/α cell
α2
α1
a1
α2
a1
RME1
meiosis

1.3b Entry into the Sporulation Pathway: Role of the RME1 Gene

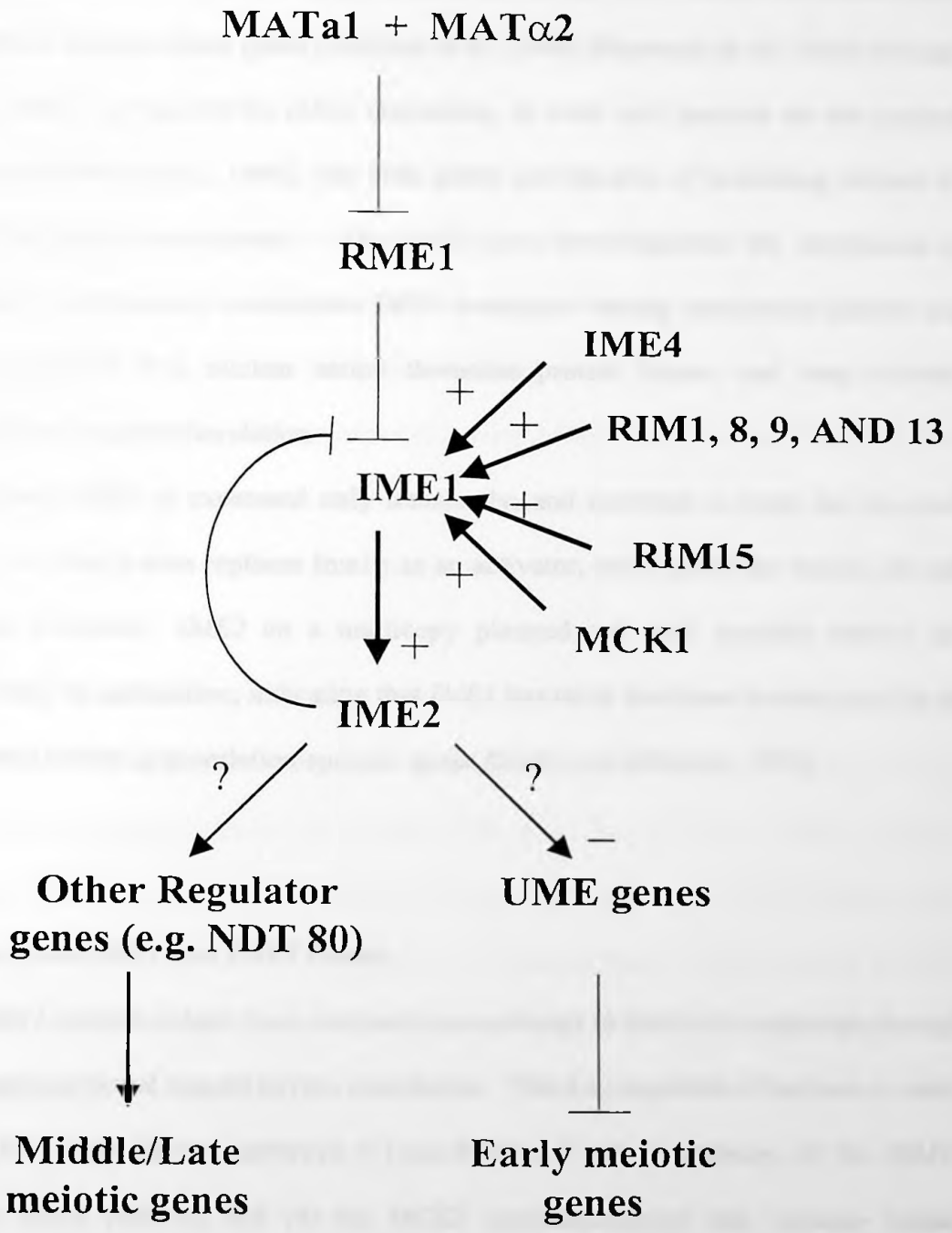
The *RME1* gene is expressed in the homozygous diploids **a**/**a** and α/α and in haploid cells but not in heterozygous diploid **a**/α cells (Herskowitz et al., 1981). Diploids which are homozygous for the *rme1-1* mutation sporulate regardless of their mating type. From these and other findings (Mitchell and Herskowitz, 1986), it appears that *RME1* is an inhibitor of sporulation and that **a**/α cells enter the sporulation pathway by using the a1/α2 repressor to turn off the *RME1* gene (Strich et al., 1989). *RME1* transcripts levels are higher in haploid cells compared to diploid **a**/α cells indicating that the haploid specific gene *RME1* is transcriptionally regulated by the a1/α2 repressor. Rme1p represses transcription of *IME1* which is an early positive regulator of sporulation. Repression of *RME1* by the a1/α2 repressor allows expression of *IME1* and subsequent induction of *IME1*-dependent sporulation-specific genes (Fig. 4 and Fig. 5).

1.3c The *IME1* and *IME2* Genes

The *IME1* gene was cloned by its high copy number effects which enable MAT insufficient (haploid and diploid homozygous) cells to undergo meiosis. *ime1* null mutants are blocked in premeiotic DNA synthesis (Kassir et al., 1988) and lead to undetectable levels of early, middle and late meiotic transcripts such as *IME2*, *SPO13*, *SPS1* and *SPS2* (Mitchell et al., 1990; Yoshida et al., 1990). *IME1* expression in vegetative cells is very low compared to 5 to 30-fold higher levels observed in nitrogen-starved **a**/α cells (Kassir, 1988; Smith and Mitchell, 1989). This evidence strongly suggests that *IME1* is a positive regulator of meiosis (Kassir et al.,

Figure 5: Genetic Control of Sporulation. Genetic control is conferred by the mating type locus (MAT). a1/α2 repressor made by heterozygous diploid cells represses the expression of *RME1* and allows for the expression of the key regulator of meiosis *IME1*. *IME1* activates *IME2* and other early sporulation-specific genes directly and activates middle and late genes indirectly through *IME2*. Timely and uninterrupted expression of these genes culminates in the process of meiosis and formation of four recombinant haploid ascospores in an ascus. *IME1* is regulated by four distinct pathways involving *RIM1, 8, 9 and 13*, *IME4*, *RIM15* and *MCK1*.

GENETIC CONTROL



1988). The actual function of *IME1* will be discussed below in connection with the *UME6* gene. *IME2* is an early sporulation-specific gene that is required for entry into sporulation (Fig. 5). Ime2p is a positive regulator of middle and late meiotic genes, e.g. *SPS1* and *SPS2,* as *ime2* null mutants are unable to express these genes (Mitchell et al., 1990; Hepworth et al., 1995; Sia and Mitchell, 1995). *IME1* is required for *IME2* expression, as *ime1* null mutants do not express *IME2* transcripts (Mitchell et al., 1990), but both genes are capable of activating subsets of sporulation-specific genes independently. The *IME2* gene downregulates the expression of *IME1* because *ime2* null mutants overexpress *IME1* transcripts during sporulation (Smith and Mitchell, 1989). Ime2p is a nuclear serine threonine protein kinase and may activate downstream regulators by phosphorylation.

In one model, *IME1* is expressed only transiently, and activates a small set of genes including *IME2*. As Ime2p then replaces Ime1p as an activator, early genes are turned off and middle genes are activated. *IME2* on a multicopy plasmid can only partially relieve the requirement for *IME1* in sporulation, indicating that *IME1* has other functions besides turning on the *IME2*-dependent subset of sporulation-specific genes (Smith and Mitchell, 1989).

1.3d Regulators of the *IME1* and *IME2* Genes

Several *IME1* regulators have been analyzed in an attempt to define the pathways through which cell type and nutritional signals govern sporulation. This key regulator of meiosis is under the regulation of four main distinct pathways: (1) the *RIM 1,8,9* and *13* pathway, (2) the *RIM15* pathway, (3) the *IME4* pathway and (4) the *MCK1* (serine/threonine and tyrosine kinase) pathway. Analysis of *rim mck1*, *rim ime4* and *rim rim* double mutants indicate that these

pathways act independently as *rim mck1* and *rim ime4* mutants have more severe sporulation defects than any of the single mutants (Li and Mitchell, 1997). Studies with *rim rim* double mutants indicate that the Rim proteins (Rim1p, 8p, 9p and 13p) act together in a protein complex or in successive steps, as the defects in the *rim* single mutant are similar to the double mutant (Li and Mitchell, 1997).

RIM15, another regulatory gene, was identified through a mutation that reduced the ability of *S. cerevisiae* cells to undergo meiosis; it is believed to affect activity of the Ime1p-Ume6p-Rim11p pathway by stimulating Ime1p-Ume6p interactions. It is not known if the role of Rim15p in Ime1p-Ume6p complex formation is direct or indirect, even though *IME1* expression is reduced in *rim15Δ/rim15Δ* diploids (Vidan and Mitchell, 1997), and Ime1p-Ume6p interactions in a two-hybrid assay diminish in *rim15* mutants (Rubin-Bejerano et al., 1996). It is possible that glucose repression of Rim15p is responsible for inhibition of the Ime1p-Ume6p interactions.

It is thought that the activity of a third regulator, *IME4,* is stimulated indirectly by the a1/α2 repressor. *IME4* is expressed at higher levels in starved cells than in growing cells, and may transmit both cell type and nutritional signals (Shah and Clancy, 1992). *MCK1,* another positive regulator of *IME1,* encodes a protein kinase homolog that cofractionates with serine/threonine and tyrosine kinase activities. Null *mck1* mutants lead to partial defects in *IME1* expression and sporulation, suggesting the existence of parallel pathways regulating *IME1* expression (Su and Mitchell, 1993).

1.4 Role of *UME* Genes in Early Gene Regulation

UME (unscheduled meiotic gene expression) genes (*UME1-6*) are a group of regulators of early meiotic genes (Fig. 5). *ume* mutants were first isolated in a screen in which early meiotic gene (*SPO13*) derepression in vegetative cells was detected by measuring *SPO13-lacZ* reporter gene expression. Loss of function of *UME1-5* genes showed a 2- to 10-fold increase of early meiotic transcripts in vegetative cells, while loss of *UME6* function results in a greater increase (Strich et al., 1989). Based on a number of studies (Strich et al., 1989, 1994 and Rubin-Bejerano et al., 1996), *UME* genes are believed to act downstream of cell type and nutritional control and participate in a regulatory cascade which causes transcriptional activation of meiotic genes.

1.4a The *UME6* Gene

Loss of *UME6* function leads to a 70-to 100-fold increase in early meiotic transcripts (e.g., *SPO11* and *SPO13*) during vegetative growth which suggests that Ume6p is a negative regulator of early meiotic genes during mitosis. *UME6* mRNA is constitutively transcribed during mitosis and meiotic development, which suggests that Ume6p activity is posttranscriptionally regulated (Strich et al., 1994). Although transcription of meiotic genes is derepressed in *ume6* mutants during mitosis, generation times of haploid and diploid cells are barely reduced. However, ascus formation and spore viability are severely reduced in *ume6* mutants (Strich et al., 1994). The *UME6* gene encodes a 91kD protein that contains C6 Zinc cluster motifs that are similar to the DNA binding domain of GAL4.

URS1 is a repressor binding site found in both early meiotic (e.g. *SPO13*, *HOP1*, *SPO11*

and *IME2*) and non-meiotic genes (e.g. genes involved in carbon and nitrogen metabolism, inositol metabolism, heat shock response and electron transport) (Luche et al., 1992; Park et al., 1992). Several genes that are under *UME6* control have an URS1 site in their 5' noncoding regions. Ume6p appears to regulate expression of these genes through the URS1 element, even though not all genes containing URS1 sites in their 5' regulatory region are regulated by Ume6p (Strich et al., 1994). The specificity of Ume6p/URS1 repression is believed to occur through association with additional proteins. For example, Ume6p and a heteromeric protein named binding URS1 factor (BUF), composed of 37.5kD and 73.5kD monomers, are believed to bind URS1 (Luche et al., 1992; Park et al., 1992). URS1 is a repressor site in cells lacking Ime1p and an activator site in cells expressing Ime1p. Repression through URS1 is dependent on *UME6* and activation is achieved by *IME1*-dependent conversion of *UME6* into an activator (Bowdish et al., 1994).

To date, two upstream elements are known that mediate early meiotic gene expression in cooperation with URS1: (1) the T_4C site of the *IME2* gene, and (2) the UAS site of the *HOP1* gene. Bowdish and Mitchell (1993) showed by mutational studies that both URS1 and T_4C have positive roles in the regulation of the *IME2* gene. Both the T_4C site and the URS1 site are weak upstream activation sites in isolation. URS1 activation is *IME1*-dependent while T_4C is *IME1*-independent and responds to carbon sources. These observations suggest a model in which T_4C is not required for URS1 to respond to *IME1*, but T_4C amplifies the *IME1*-dependent activation signal in these sporulation specific genes (Bowdish et al., 1995).

Recently, two-hybrid studies by Rubin-Bejerano et al. (1996) showed that Ume6p is converted from a repressor during vegetative growth into an activator during sporulation. In this model, Ime1p and Ume6p join to become a complete transcriptional activator complex in which

Ume6p provides the DNA binding domain and Ime1p provides a transactivation domain. Ime1p lacks an identifiable DNA binding domain and Ume6p lacks an activation domain. This study also showed that during sporulation Ime1p forms homodimers, which are subsequently phosphorylated by a protein kinase, Rim11p; Ime1p is then recruited by Ume6p to the 5' regulatory region of URS1-containing genes. Interactions between Ime1p and Ume6p position the activation domain of Ime1p correctly, allowing the complex to function as an activator during sporulation.

In *ume6* mutant strains, transcript levels of *SPS4*, a middle gene, are not derepressed during mitosis as are early sporulation-specific gene transcripts. This finding suggests that a pathway distinct from that regulating early genes, regulates *SPS4* and other middle and/or late genes. The absence of a region with homology to the URS1 element in most of these genes suggests that they are less likely to be directly regulated by *UME6* (Hepworth et al., 1995).

1.4b The *UME1-5* Genes

Meiotic mRNA abundance is regulated by both transcriptional initiation and RNA turnover. Although induction of transcript initiation is the predominant mechanism of transcript accumulation, a rapid RNA degradation system is believed to operate to maintain very low basal transcript levels during vegetative growth and some stages of sporulation. For example, studies on deletion derivatives of the early gene *SPO13* suggest that determinants located within the coding region contribute to the high instability of this transcript during vegetative growth and sporulation (Surosky and Esposito 1992). Besides these built-in determinants, regulatory proteins also participate in maintaining the stability (or instability) of the transcripts.

Two of the *UME* genes, *UME5* and *UME2,* act in the same pathway to regulate meiotic transcript stability (Surosky et al., 1994). A *GAL4-SPO13* transcriptional fusion was used to measure *SPO13* mRNA half-life by inducing it in galactose medium and repressing it in glucose medium. The mRNA levels were determined at different time points using a S1 nuclease protection assay. The *GAL4-SPO13* mRNA was two-fold more stable in *ume2* and *ume5* mutants compared to the wild type strain (Surosky et al., 1994).

The *UME5* transcript is constitutively expressed and encodes a predicted 63kD protein. It decreases meiotic transcript stability two-fold during vegetative growth but does not affect the stability of the mitotic transcripts under the same conditions. This pathway is independent of the cis-acting determinants that alter mRNA stability. There is some evidence to indicate that *UME5* is a serine/threonine kinase. First, a sequence homology search using the algorithm BLASTP revealed significant homology to the *CDC28* family of serine/threonine protein kinases. Second, alteration of a highly conserved lysine in the putative kinase domain leads to an increase in cell generation time, decrease in sporulation efficiency, and a two-fold increase in the mitotic stability of the *GAL4-SPO13* mRNA in a *ume5* mutant strain. Kinase activity must be central to the function of the *UME5* gene, as mutations of the conserved amino acids give a phenotype identical to the *ume5* deletion phenotype (Surosky and Esposito, 1994). Based on this evidence, *UME5*-mediated mRNA stability involves phosphorylation of a substrate(s) which in turn, directly or indirectly, leads to more rapid mRNA turnover (Surosky et al., 1994).

1.5 Control of Middle Sporulation-Specific Genes.

The early regulatory genes *IME2* and *MCK1* have been shown to play a role in the

regulation of middle and late genes. A new gene, *IDS2* (*IME2*-dependent signaling) with functional relationship to *IME2,* has been identified. An *ids2* null mutant delays the downregulation of *IME1* and expression of middle and late genes. Ids2p is dispensable for expression of early genes *HOP1* and *SPO13,* but is essential for the expression of middle and late genes *SPS1*, *SPS2* and *SPS100* in a *ime1* null mutant strain (Sia and Mitchell, 1995).

1.5a Significance of the MSE

Mid sporulation elements (MSE) found in the 5' regulatory region of middle sporulation and other sporulation genes including *NDT80*, *SPS4*, *SGA1, SPR3, SPR6* and *SPR28* (Hepworth et al., 1995; Ozarac et al., 1997) are believed to play a role in the *NDT80*-dependent transcriptional regulation. The consensus MSE sequence was defined as gNCRCAAA(A/T) by Ozsarac et al. (1997). In the middle gene *SPR3,* the 9 bp MSE is located within a palindrome essential for the developmental regulation of the gene.

1.5b The NDT80 Gene

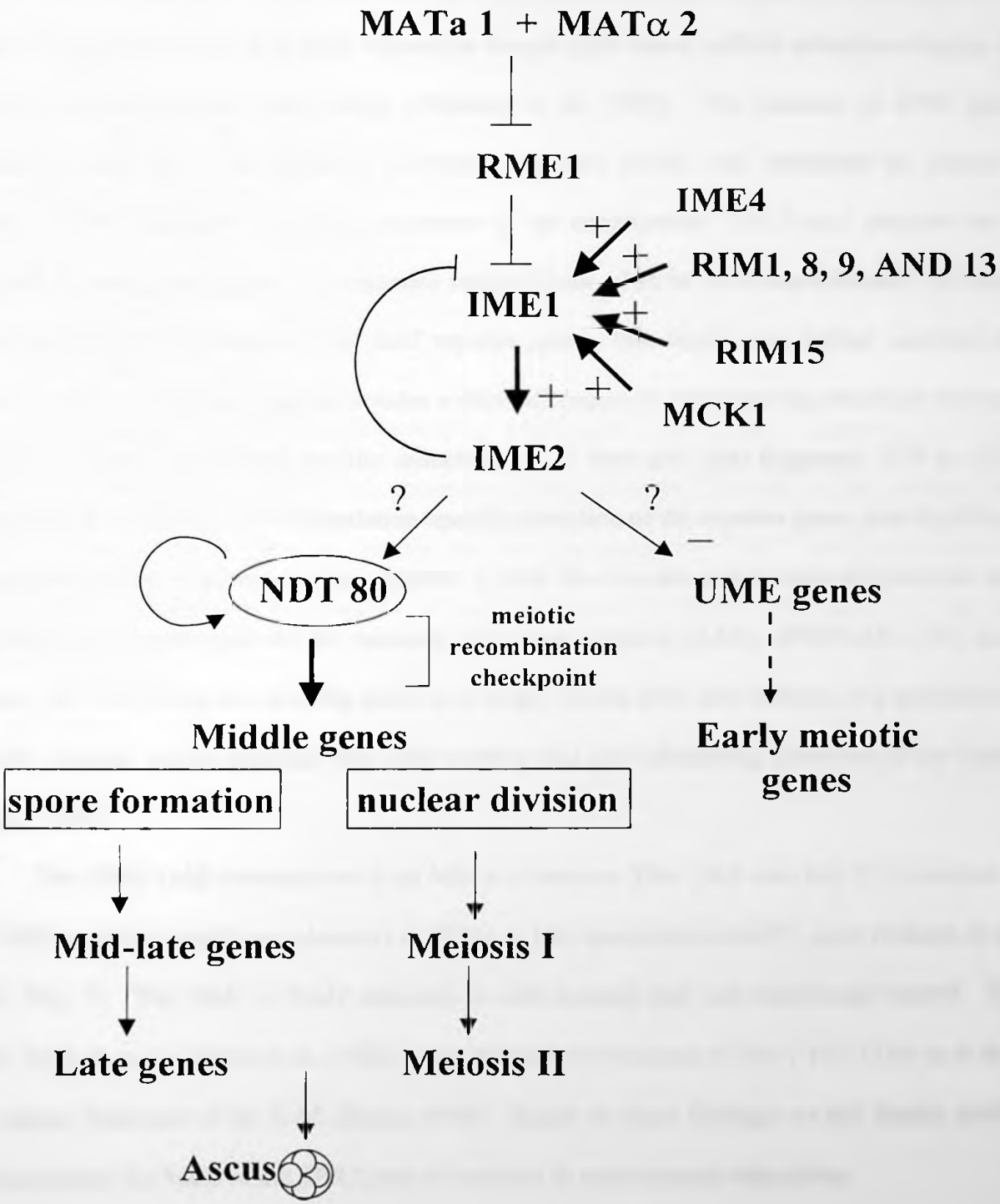
The *NDT80* (non dityrosine) gene was identified in a screen for mutants defective in spore wall formation. First, mutants that could not alleviate the spore formation defect by elimination of the *SPO11* gene (a gene required for recombination) were identified. Further screening revealed one mutant, *ndt80-1,* that was completely defective in spore formation and arrested at pachytene stage with full-length synaptonemal complexes (SC) and duplicated, unseparated spindle polar bodies (SPB). Analysis of *NDT80* coding region revealed that it

encodes a 627 amino acid protein with positively (13%) and negatively (14.1%) charged amino acids. It also contains a total of 101 serine and threonine residues; however, no sequences within the protein match consensus sequences for phosphorylation targets of PKA, PKC or CDC28. No significant homology was found between *NDT80* sequences and other known proteins in the GenBank Database (Xu et al., 1995).

Recently, Ndt80p has been shown to recognize MSEs and to activate transcription of middle sporulation-specific genes based on the following evidence: (1) *NDT80* is required for middle sporulation-specific RNA synthesis; (2) ectopic expression of *NDT80* during mitosis activates expression of middle sporulation-specific genes; (3) Ndt80p recognizes the MSE sequence in gel shift assays, and (4) Ndt80p activation is dependent on conserved base pairs of the MSE (Chu and Herskowitz, 1998). Even though there is a striking correlation between the presence of the MSE and Ndt80p dependent expression, a perfect fit to the consensus sequences is not required. *NDT80* transcription occurs after expression of early genes like *IME1* and *IME2* expression, and is dependent on *IME1* expression. *NDT80* in turn activates transcription of middle genes and is involved directly or indirectly in the expression of mid-late genes such as *DIT1* (Fig. 6). The promoter of *NDT80* contains an MSE, and Ndt80p has been shown to promote its own transcription. *NDT80* is transcribed later than early genes, but somewhat earlier than middle genes, and is considered a "delayed early gene" (Chu and Herskowitz, 1998; Clancy, 1998).

Figure 6: Regulation by *NDT80* of Middle and Late Genes. *NDT80*, a delayed early gene, is a positive regulator of middle and late genes. *ndt80* mutants arrest at the pachytene stage of meiosis I and are unable to express middle and late sporulation-specific genes.

GENETIC CONTROL



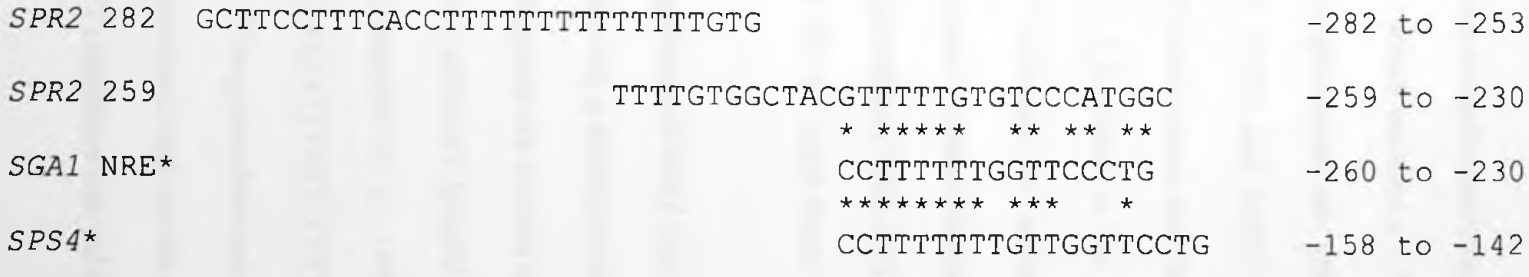
1.6 The Late Sporulation-Specific Genes

1.6a *SPR2*

The *SPR2* gene has been classified as a late sporulation-specific gene as it is expressed six hours into sporulation with peak expression around eight hours; mRNA abundance begins to decline between ten and twelve hours (Holaway et al., 1985). The function of *SPR2* gene product is unknown. An upstream activation sequence (UAS) was identified by inserting different *SPR2* promoter fragments upstream of an enhancerless *CYC1-lacZ* reporter on a replicating (multicopy) vector. A sequence ranging from -295 to -210 was sufficient to confer sporulation-specific induction of the *lacZ* reporter gene. This region was further analyzed by making small overlapping oligonucleotides within this region to determine the minimum element required to confer sporulation-specific induction. A 31 base pair (bp) fragment, -259 to -230, was shown to be sufficient for sporulation-specific induction of the reporter gene, was capable of maintaining proper regulation of the reporter in both the forward and reverse orientations, and thus satisfy the requirement for an upstream activation sequence (UAS). *SPR2*UAS-*CYC1-lacZ* fusions that were integrated into the genome as single copies were also induced in a sporulation-specific manner, which indicated that copy number was not influencing induction of the fusion (Riggs, 1994).

The *SPR2* UAS contains two 9 bp MSEs in tandem. This UAS also has 9/10 matches to the NRE (negative regulatory element) of *SGA1*, a late sporulation-specific gene (Kihara et al., 1991; Fig. 7). The NRE of *SGA1* responds to both mating type and nutritional control. The *SPR2* MSE does not function as a NRE when inserted downstream of the *CYC1* UAS as it does not repress induction of the UAS (Riggs, 1994). Based on these findings we did further studies to characterize the MSE of the *SPR2* gene as outlined in experimental objectives.

Figure 7: Alignment of Upstream Sequences of Middle and Late Genes. Alignment of *SPR2* upstream sequences with the NRE (negative regulatory element) of *SGA1*, a late sporulation-specific gene, and with the UAS (upstream activation sequence) of *SPS4*, a middle sporulation-specific gene. Matches are indicated by *. Coordinates are numbered with respect to the first ATG of each gene.

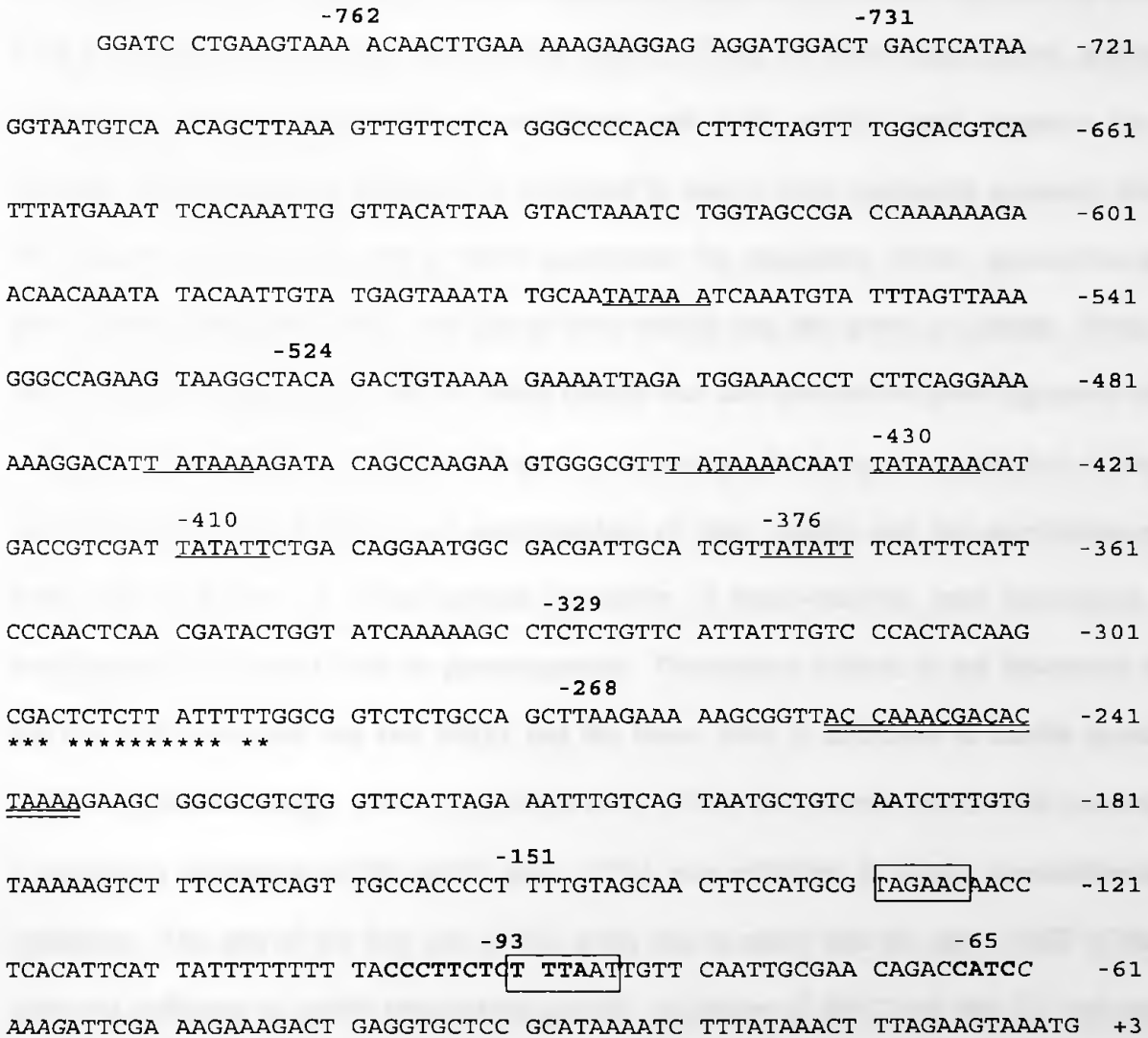

SPR2 282 GCTTCCTTTCACCTTTTTTTTTTTTTTGTG -282 to -253
SPR2 259 TTTTGTGGCTACGTTTTTGTGTCCCATGGC -259 to -230
SGA1 NRE* CCTTTTTTGGTTCCCTG -260 to -230
SPS4* CCTTTTTTTGTTGGTTCCTG -158 to -142

1.6b *SPR1* (*SSG1*)

The purpose of this study was to understand the regulation of the late sporulation-specific gene *SPR1* (also known as *SSG1),* which encodes a 1,3-β-glucanase (San Segundo et al., 1993). Even though several endo- and exoglucanases are present during vegetative growth, only two constitutive β-glucanases, namely *EXG1* and *EXGII,* have been studied extensively. These forms are first secreted to the periplasmic space and later released into the culture medium (San Segundo et al., 1993). Because 1,3-β-glucan is the main polysaccharide responsible for the strength and integrity of the cell envelope, the enzymatic activity of the 1,3-β-glucanases is thought to be necessary for morphogenic events that require controlled hydrolysis of the cell wall (San Segundo et al., 1993). In accordance with its possible role in ascospore packaging, the *SPR1* gene is expressed around six to eight hours after transfer into sporulation medium (Muthukumar et al., 1993).

The 1,335 bp open reading frame of *SPR1* has a predicted coding capacity of 445 amino acids that specify an acidic protein with a hydrophobic leader sequence. Its promoter region is unusual in that canonical TATA elements are located at -441, -471 and -565, whereas the TATA elements in most other *S. cerevisiae* genes are located between 40 to 120 bp upstream from the transcriptional initiation site (San Segundo et al., 1993). The *SPR1* promoter also has a long pyrimidine-rich tract (TTTTTTTTACCCTTCTCTTTT) between -108 and -89 followed by a CAAAG sequence at -65 (Fig. 8). The pyrimidine-rich tract and CAAG sequence (not CAAAG) in other genes usually confer constitutive, high levels of expression (San Segundo et al., 1993); the role of these sequences in *SPR1* regulation has yet to be determined.

Figure 8: The *SPR1* Promoter Sequence. Canonical TATA boxes are underlined. Transcriptional start site is at -65 and marked in bold letters. End points of nested deletions are shown in bold numbers. Row of asterisks indicates 10/15 match to *SPS4* UAS. Sequence with matches to the MSE is double underlined. Functional variants of TATA elements are underlined. A pyrimidine-rich tract (CT block) is in bold (-108 to -89) followed by a CAAG motif 26 bp after the CT block. Sequences with weak matches to the TATA element between -151 and +1 are in boxes.


-762
-731
GGATC CTGAAGTAAA ACAACTTGAA AAAGAAGGAG AGGATGGACT GACTCATAA -721
GGTAATGTCA ACAGCTTAAA GTTGTTCTCA GGGCCCCACA CTTTCTAGTT TGGCACGTCA -661
TTTATGAAAT TCACAAATTG GTTACATTAA GTACTAAATC TGGTAGCCGA CCAAAAAAGA -601
ACAACAAATA TACAATTGTA TGAGTAAATA TGCAATATAA ATCAAATGTA TTTAGTTAAA -541
-524
GGGCCAGAAG TAAGGCTACA GACTGTAAAA GAAAATTAGA TGGAAACCCT CTTCAGGAAA -481
-430
AAAGGACATT ATAAAAGATA CAGCCAAGAA GTGGGCGTTT ATAAAACAAT TATATAACAT -421
-410
-376
GACCGTCGAT TATATTCTGA CAGGAATGGC GACGATTGCA TCGTTATATT TCATTTCATT -361
-329
CCCAACTCAA CGATACTGGT ATCAAAAAGC CTCTCTGTTC ATTATTTGTC CCACTACAAG -301
-268
CGACTCTCTT ATTTTTGGCG GTCTCTGCCA GCTTAAGAAA AAGCGGTTAC CAAACGACAC -241
*** ********** **
TAAAAGAAGC GGCGCGTCTG GTTCATTAGA AATTTGTCAG TAATGCTGTC AATCTTTGTG -181
-151
TAAAAAGTCT TTCCATCAGT TGCCACCCCT TTTGTAGCAA CTTCCATGCG TAGAACAACC -121
-93
-65
TCACATTCAT TATTTTTTTT TACCCTTCTC T TTAATTGTT CAATTGCGAA CAGACCATCC -61
AAAGATTCGA AAGAAAGACT GAGGTGCTCC GCATAAAATC TTTATAAACT TTAGAAGTAAATG +3

Hypothesis

Expression of developmentally regulated genes is controlled by a promoter and other specific regulatory sequences. Complete regulation requires interaction between the promoter, RNA polymerase, and general transcription factors (TFIIs) for basal transcription, and specific interactions between transcriptional regulators and both positive and negative cis-acting elements. Developmental regulation is conferred by one or more regulatory sequence elements. The purpose of this study was to better understand the regulation of two sporulation-specific genes, *SPR1* (*SSG1*) and *SPR2*, and that of other middle and late genes in general. These genes were chosen because little is known about middle and late sporulation gene regulation whereas early gene regulation has been studied quite extensively. Studying the regulation of the *SPR1* and *SPR2* genes will facilitate our understanding of other middle and late sporulation-specific genes and shed light on transcriptional regulation of tissue-specific gene expression during developmental processes such as gametogenesis. Preliminary studies in our laboratory suggest that the *SPR2* promoter has two MSEs and the lower MSE is sufficient to confer sporulation-specific regulation (Riggs, 1994). Hepworth et al. (1995) have shown that a MSE present in the 5' regulatory sequences of the middle gene, *SPS4*, was sufficient to confer sporulation-specific regulation. The aim of the first part of this study was to show that the upper MSE of the *SPR2* gene was sufficient to confer sporulation-specific regulation of *SPR2* and that GC rich region of the MSE is required for maximal induction. The next part of this study was aimed at determining the mode of regulation of *SPR1* by mapping the location of the promoter and other cis-acting elements necessary for sporulation-specific expression. The experimental approach taken to answer each specific aim is described below.

1.7 Experimental Objectives

1.7a *SPR2*

1) The role of putative MSE elements in *SPR2* regulation was tested by making *SPR2-CYC1-lacZ* transcriptional fusions and measuring β-galactosidase activity in vegetative and sporulating GKY5 cells.
2) The significance of the GC bases in the lower MSE was determined by mutagenesis of these bases and measuring the effect on expression of an MSE-*CYC1-lacZ* reporter.
3) The role of Ime2p, Ume6p and Ndt80p was determined by measuring *SPR2-CYC1-lacZ* expression in strains mutant for these proteins.
4) Protein-DNA interactions between the *SPR2* regulatory sequence and sporulation-specific transactivators were determined by electrophoretic mobility shift assays (EMSAs). Interaction between Ndt80p and *SPR2* sequences was established by EMSAs using recombinant Ndt80p.

1.7b *SPR1*

1) The *SPR1* gene lacks a canonical TATA element. The basal *SPR1* promoter element was mapped by fusing the *SPR2* MSE to *SPR1* promoter fragments which were fused to the *lacZ* reporter.
2) The 5' regulatory region of *SPR1* was mapped by making a series of 5' nested *SPR1-lacZ* translational fusions. The presence of sporulation-specific regulatory sequences was assessed by the loss of activity of nested *SPR1-lacZ* fusions during sporulation.

3) Significance of candidate sequences was determined by making "window" deletions within the promoter of *SPR1-lacZ* fusions and by comparing these candidates to known cis-acting regulatory elements.

4) Sequences with substantial effects on expression of the *SPR1-lacZ* reporter were tested for UAS function by making *SPR1-CYC1-lacZ* transcriptional fusions. These sequences were tested in both forward and reverse orientations to determine if they are UAS elements.

5) The role of Ime2p, Ume6p and Ndt80p in the sporulation-specific regulation of *SPR1* was tested by measuring the expression of *SPR1-lacZ* fusions in corresponding mutant strains.

6) DNA-protein interactions between *SPR1* promoter sequences and sporulation-specific proteins were determined by EMSAs. Interactions between an SPR1 probe and proteins present in vegetative and sporulating crude extracts from wild type cell were compared. The role of Ndt80p was assessed by comparing complex formation in wild type and *ndt80* mutant strains and by testing for interactions between recombinant Ndt80p and an *SPR1* promoter element.

2.0 RESULTS

2.1 Regulation of the *SPR2* Gene

2.1a Functional Significance of the Upper MSE

Studies in our laboratory suggested that the *SPR2* UAS (-259 to -230) is a composite element consisting of two exact copies of TTTTTGTG followed by GC rich sequences GCTACG and TCCCATGGC, respectively (Riggs, 1994; Fig. 7). A promoter fragment containing both the elements confers sporulation-specific regulation to a *CYC1-lacZ* reporter gene in both the forward (reporter SPR2/259F) and reverse (reporter SPR2/259R) orientations. Reporter SPR2/259F is induced about 100-fold during sporulation (Table 2). In order to determine if either of these copies was sufficient to confer sporulation-specific regulation or UAS function, each repeat was inserted separately into the enhancerless *CYC1-lacZ* reporter (Table 2). These sequences were expected to induce β-galactosidase expression in sporulating cells and not in vegetative cells, if they are sporulation-specific activation sequences. The lower element (reporter SPR2/R2) is capable of sporulation-specific induction (33-fold) in both the forward and reverse orientations, strongly supporting the argument that the lower element has enhancer-like function (Riggs, 1994).

The upper element was tested in both the forward (reporter SPR2/R1F) and reverse (reporter SPR2/R1R) orientations. β-galactosidase levels for the upper element in the forward orientation (SPR2/R1F) during vegetative growth and sporulation were 5.0 Miller units/mg protein and 109.0 Miller units/mg protein, respectively, which represents a 22-fold induction; comparable induction was detected in the reverse orientation for SPR2/R1R (Table 2). These data support the hypothesis that the upper element is also an UAS. Because of these structural

Table 2: Analysis of the *SPR2-CYC1-lacZ* Transcriptional Fusions. These assays were performed in Strain GKY5 (wild type). The *SPR2* MSE was tested by making a series of constructs containing different portions of the regulatory element. The SPR2/259 constructs contain both the upper and lower MSEs; SPR2/R1 and SPR2/R2 constructs contain either the upper or lower MSEs, respectively. TCGA indicates the overhang used for subcloning purposes. β-galactosidase activities shown are mean values of three or more assays. Expression of β-galactosidase reporter was quantified and reported as Miller units with standard deviations as described. Fold induction (Fold) was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement. F and R indicate forward and reverse orientation, respectively.

		β-galactosidase activity (Miller units/mg protein)		
Reporter	**Sequence Tested**	**PSP_2**	**SP_2**	**Fold**
SPR2/259F	TCGATTTTTGTGCTACGTTTT TGTGTCCCATGG	12.8 ±1.3	939.51 ± 219.0	105
SPR2/259R	CCATGGGACACAAAACGTAG CACAAAAATCGA	7.4 ± 1.6	1061.7 ± 8.49	183
SPR2/R1F (upper MSE)	TCGATTTTTGTGCTACGTa	4.9 ± 1.0	109.0 ± 14.9	22
SPR2/R1R (upper MSE)	tACGTAGCACAAAATCGA	10.9 ±2.0	163.0 ± 10.7	15
SPR2/R2F (lower MSE)	TCGAGTTTTGTGTCCCATGG	3.2	104.5	33
SPR2/R2R (lower MSE)	CCATGGGACACAAAACTCGA	4.7	113.7	24
SPR2/R2FdraI (lower mutant)	TCGAGTTTTTGTGTttaAaGG	7.8 ± 2.4	29.6 ± 14.2	4
SPR2/R2RdraI (lower mutant)	CCtTtaaCACAAAAACTCGA	7.7 ± 2.0	22.4 ± 5.9	3
SPR2/R2FbglII (lower mutant)	TCGAGTTTTTGTGTaagATct	3.6 ± 3.9	47.4 ± 5.4	13

and functional similarities to the mid sporulation element, these tandem elements will be designated the upper MSE and lower MSE, respectively.

The level of induction observed with the composite element (reporter SPR2/259F) was more than 5-fold higher than that observed with either element alone (SPR2/R2F or SPR2/R1F). This suggests that the effect of the tandem elements is synergistic rather than additive.

2.1b Significance of the Adjacent GC Sequences

In order to determine the importance of the GC rich region at the 3'ends of the lower MSE, this region was replaced with sequences that were AT rich (Table 2). The first set of mutations was made by substituting the CCCATGG sequence (-235 to -228) with ttaAaGG and a second set of mutations was made by substituting CCCATGG with aagATct. These mutations also served as DraI and BglII restriction sites to confirm the presence of the insert. The expression of mutant MSE-*CYC1-lacZ* constructs was monitored in both vegetative and sporulating cells (Table 2). The SPR2/R2FdraI reporter activity was 7.8 Miller units/mg protein and 29.6 Miller units/mg protein in vegetative and sporulating cells, respectively, which represents approximately a 4-fold induction. This finding suggests that the mutant MSE is still properly regulated during vegetative growth but that the level of induction during sporulation is reduced compared to SPR2/R2F. The SPR2/R2RdraI reporter contains the mutant MSE in the reverse orientation and behaves very similarly to the SPR2/R2FdraI reporter (7.7 Miller units/mg protein and 22.4 Miller units/mg protein in vegetative and sporulating cells, respectively). The induction level of the forward and reverse fusions is 28% and 20% that seen with the wild type reporters (SPR2/R2F and SPR2/R2R). Taken together these findings suggest that the GC rich

region at the 3' end of the lower element is required for full induction by the *SPR2* MSE.

As seen in Table 2, the BglII (SPR2/R2bglII) mutant of the same sequence showed β-galactosidase levels similar to the wild type fusion during vegetative growth. However, induction during sporulation was reduced to 47.42 Miller units/mg protein, which represented a 13-fold induction. Similar to the DraI (SPR2/R2draI) mutants, the BglII mutant was able to maintain sporulation-specific regulation of the reporter, but was not able to maintain the same level of induction as the wild type, even though it gave higher induction than DraI (SPR2/R2draI) mutants. These results are consistent with the requirements of the GC rich region for full sporulation-specific induction.

2.1c Effect of Specific Regulators on *SPR2* Expression.

Three regulatory proteins, Ime2p, Ume6p and Ndt80p, control the expression of middle and late sporulation genes. Ime2 is an early sporulation-specific gene which encodes a nuclear serine threonine protein kinase (Yoshida et al., 1990; Kominami et al., 1993); this gene is required for the transcriptional regulation of many other sporulation-specific genes (Kupiec et al., 1997). Ume6p is a transcription factor which functions as a repressor of early sporulation-specific genes during vegetative growth, but functions as a positive regulator of early meiotic genes in sporulation cells (Bowdish and Mitchell, 1993; Strich et al., 1994; Bowdish et al., 1995; Steber and Esposito, 1995). Ndt80p is a regulator of middle and late sporulation-specific genes which acts by binding to the MSE (Chu and Herskowitz, 1998). To determine if these genes are required for *SPR2* expression, *SPR2-CYC1-lacZ* reporters were introduced into wild type, *ime2*, *ume6* and *ndt80* mutant strains and assayed under both vegetative and sporulation conditions.

Reporter SPR2/400F contains the *SPR2* promoter from -400 to -210 fused to *CYC1-lacZ* gene; the promoter region carries both *SPR2* MSEs. This reporter has no detectable activity during sporulation in either *ime2* or *ume6* strains, but shows levels similar to the wild type strain during vegetative growth (Table 3). *SPR2-CYC1-lacZ* constructs containing sequences -259 to -244 were tested in the *ndt80* mutant strain because the MSE was shown to be sufficient for interaction with NDT80. Reporters SPR2/259F and SPR2/R1F in the *ndt80* mutant strain (Table 3) were expressed at levels similar to wild type levels during vegetative growth. No sporulation was observed in the mutant strain (0%) compared to wild type strain (62 to 70%). Likewise, reporter expression was not induced in the *ndt80* mutant strain. In contrast, reporter expression was repressed in the *ndt80* mutant strain with constructs SPR2/259F and SPR2/R1F in sporulation medium. Taken together these data indicate that all three regulatory proteins act as positive regulators of the *SPR2* gene.

2.1d Sporulation-Specific Protein-DNA Interactions

To determine if sporulation-specific proteins bound to the *SPR2* MSEs, EMSAs were performed using crude protein extracts from strain GKY5 and a 31bp probe from -259 to -230 (SPR2/GSS259) which contains both the upper and lower MSEs. Several complexes were observed with wild type vegetative and sporulation crude extracts. Both extracts give rise to a non-specific (NS) fast migrating complex that was not competed out with cold probe. A slow migrating vegetative complex was observed (Fig. 9, lane 2) which was also competed out with 10-fold molar excess of unlabeled probe. A slower migrating sporulation-specific complex was competed out considerably with 10-fold molar excess of unlabeled probe (Fig. 9, lanes 4 and 5;

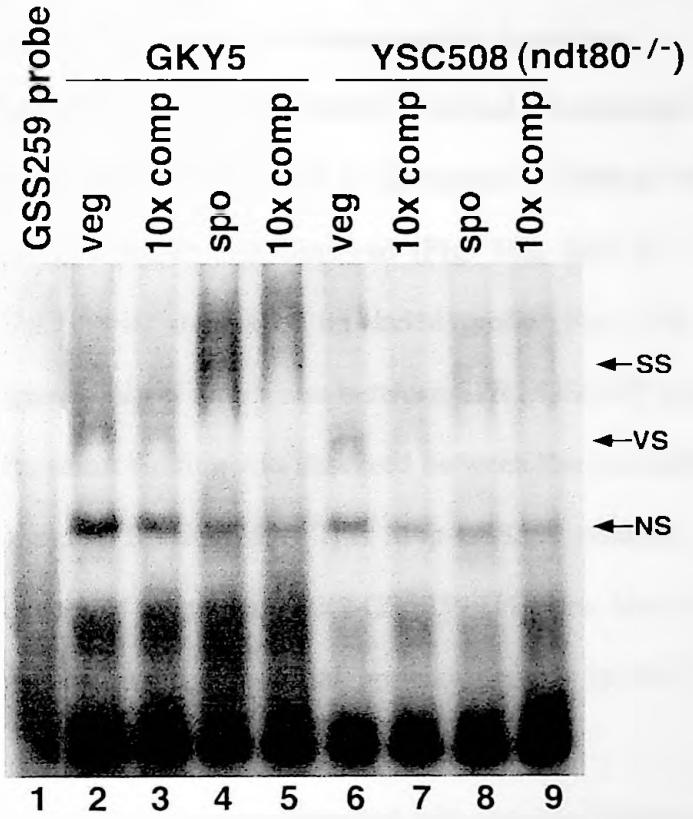
Table 3: *SPR2* Reporter Gene Expression in Strains Mutant for Sporulation-Specific Regulators. *SPR2-CYC1-lacZ* reporters were introduced in wild type or mutant strains; expression of β-galactosidase was monitored at 0 hours (PSP_2) and 12 hours (SP_2). Sporulation efficiency was determined after 24 hours in SP_2 medium. The reporters SPR2/259F (-259 to -230), SPR2/R1F (-259 to -246) and SPR2/400F (-400 to -210) were transformed into wild type (GKY5), *ndt80* (YSC508), *ime2* (AMP245) and *ume6* (RSY239) diploid **a**/α cells, respectively. ND, β-galactosidase levels < 0.01. β-galactosidase levels shown are mean values of three or more assays. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations as described. Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement. F, construct tested in the forward orientation.

β-galactosidase activity

(Miller units/mg protein)

Name	PSP_2	SP_2	% Spo.	Fold induction
Wildtype SPR2/400F	4.3 ± 0.4	619.0 ± 20.0	44%	143
ime2/SPR2/400F	3.3 ± 0.5	ND	0%	–
ume6/SPR2/400F	3.8 ± 0.9	0.5 ± 0.09	3%	0.13
Wild type SPR2/259F	12.8 ± 1.3	939.51 ± 219.0	70%	105
ndt80 SPR2/259F	13.7 ± 3.3	4.3 ± 1.1	0%	0.31
Wild type SPR2/R1F	4.9 ± 1.0	109.03 ± 14.9	62%	22
ndt80 /SPR2/R1F	9.1 ± 0.4	3.7 ± 1.5	0%	0.4

Figure 9: EMSAs with Crude Extracts and *SPR2* MSE Sequences (SPR2/GSS259). Vegetative and sporulating protein extracts were prepared from strains GKY5 and YSC508 (*ndt80* mutant). The probe alone was run in lane 1. The binding reactions contained probe and 25 μg of GKY5 vegetative extract (lanes 2 and 3), 20 μg of GKY5 sporulation extract (lanes 4 and 5), 25 μg of YSC508 vegetative extract (lanes 6 and 7), or 25 μg of YSC508 sporulation extract (lanes 8 and 9). Some reactions also contained 10-fold molar excess of unlabeled SPR/GSS259 competitor (10X comp, lanes 3, 5, 7 and 9). VS, vegetative-specific band; SS, sporulation-specific band; and NS, non-specific band which appears in both vegetative and sporulating reactions with variable intensities.


GSS259 probe
GKY5
YSC508 (ndt80⁻/⁻)
veg
10x comp
spo
10x comp
veg
10x comp
spo
10x comp
SS
VS
NS
1 2 3 4 5 6 7 8 9

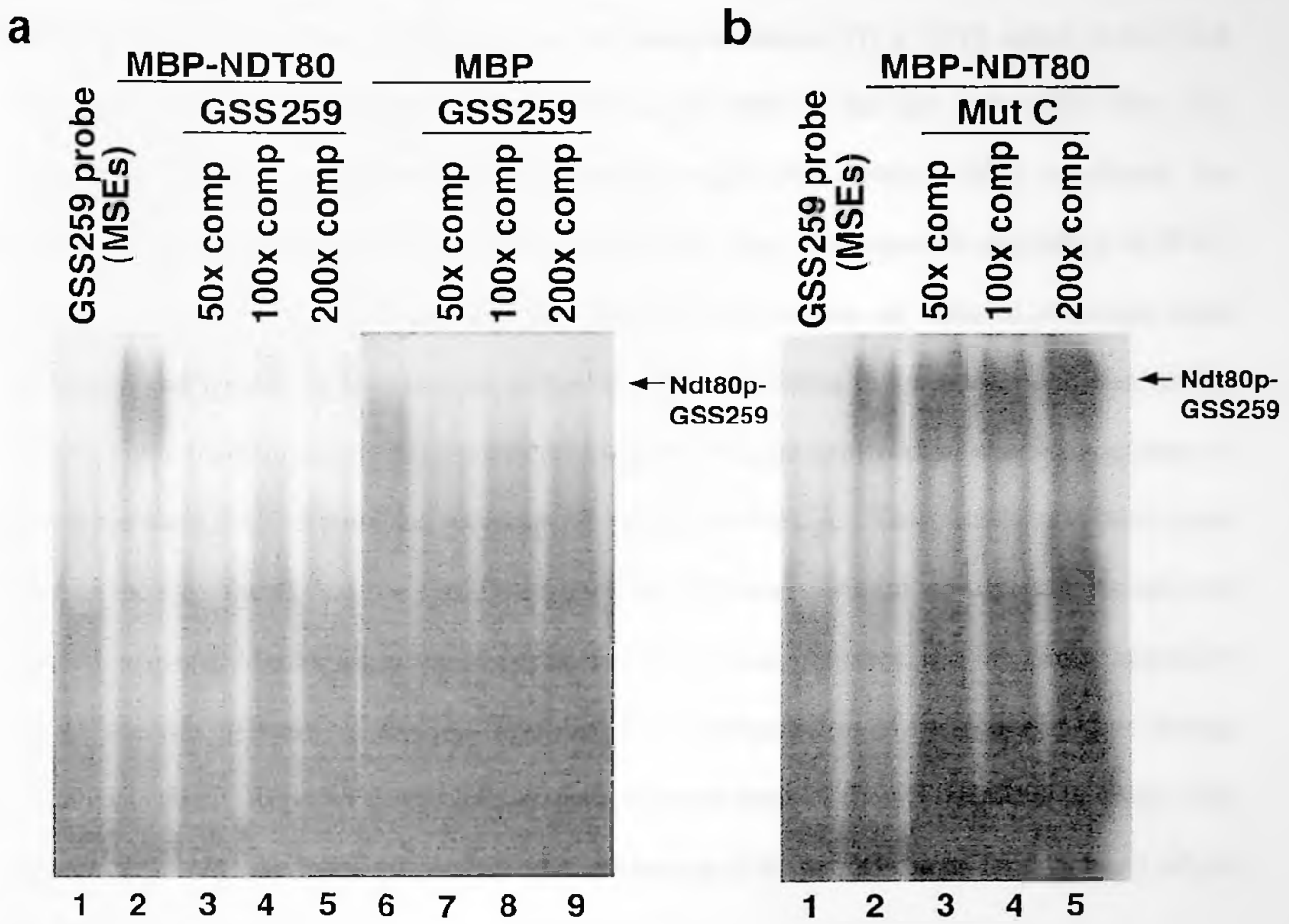
SS). Similar shift patterns were observed when EMSAs were performed with protein extracts from strain YSC508, a *ndt80* null mutant strain. However, these complexes were of lesser intensity (Fig. 9, lanes 6-9) suggesting that Ndt80p is required for the formation of the protein complex binding the DNA probe. The presence of less intense complexes with the YSC508 extracts suggests that Ndt80p is required to facilitate complex formation.

EMSAs were also performed with an affinity purified recombinant Mbp-Ndt80 fusion protein produced in bacteria and SPR2/GSS259 to determine if Ndt80p binds the *SPR2* MSE directly. A slow migrating complex was observed (Fig. 10a, lane 2). This complex was competed out with 50-fold molar excess of unlabeled probe (Fig. 10a, lane 3-5). These observations strongly suggest a direct interaction between SPR2/GSS259 (containing the MSEs) and Ndt80p. Surprisingly, some binding was observed between the recombinant Mbp (bacterial maltose binding protein) and SPR2/GSS259. This Mbp-GSS259 complex was also competed out with 50-fold molar excess of unlabeled SPR2/GSS259 (Fig.10a, lanes 6-9). This complex might arise from interactions between bacterial proteins present in the recombinant protein preparation.

The Ndt80p-MSE complex was not competed with varying molar excesses of an 11 bp mutant MSE probe (CGGTCATTGTG; MutC, mismatches to the consensus MSE sequence are underlined, Fig. 10b). Unlabeled Mut C probe in 200-fold molar excess was incapable of competing out the composite MSE probe from the complex, as the signal remained unchanged in intensity (Fig. 10b, lanes 2-5). This observation suggested that Ndt80p was bound with high affinity to the composite MSE probe and that specific nucleotides within the MSE were important to mediate the interaction between the MSE and Ndt80p.

Figure 10a: EMSAs of Ndt80p (MBP-*NDT80*) and *SPR2* MSE. Mbp-Ndt80 purified from bacteria and *SPR2* promoter sequences (SPR2/GSS259) were used in the EMSA. The probe alone was run in lane 1. All binding reactions contained probe and 2.8 μg of Ndt80p (lanes 2-5) or 2.8 μg of Mbp (lanes 6-9). Some binding reactions also contained molar excesses of unlabeled competitor (comp.) as indicated on the top panel (lanes 3-5 and 7-9). The arrow indicates the position of the Ndt80-DNA complex.

Figure 10b: Competition EMSAs with Mut C Sequence. The probe alone was run in lane 1. All binding reactions contained probe and 2.8 μg of Ndt80p (lanes 2-5). Some binding reactions also contained molar excesses of unlabeled Mut C competitor (comp.) as indicated on the top panel. Mut C is an 11 bp MSE mutant (GCCAGTAACAC); bases with mismatches to the consensus MSE are underlined. The arrow indicates the position of the Ndt80-DNA complex.


a
MBP-NDT80
GSS259
MBP
GSS259
GSS259 probe (MSEs)
50x comp
100x comp
200x comp
50x comp
100x comp
200x comp
Ndt80p-GSS259
1 2 3 4 5 6 7 8 9
b
MBP-NDT80
Mut C
GSS259 probe (MSEs)
50x comp
100x comp
200x comp
Ndt80p-GSS259
1 2 3 4 5

2.2 Regulation of the *SPR1* Gene

2.2a Search of the *SPR1* Regulatory Region

Searches of the 5' regulatory region of *SPR1* using Genepro v 4.0 revealed two sequences with weak homology to known *S. cerevisiae* regulatory elements: (1) a 10/15 match to the UAS of the middle gene *SPS4* and (2) a 12/17 match to the MSE of the late gene *SPR2* (Fig. 11). These results led us to speculate that these sequences might play a role in *SPR*1 regulation. To determine if these sequences or others were required for sporulation-specific regulation of *SPR1*, a series of nested *SPR1-lacZ* translational fusions and a series of internal deletions were constructed and tested. A translational fusion is made by joining the promoter and start codon in-frame to the reporter gene. An internal deletion is a reporter construct in which a sequence of interest has been deleted from the promoter by recombinant PCR. These constructs were made based on the hypothesis that if a significant positive regulatory element was deleted, the level of sporulation-specific induction of the reporter would decrease. Further, if a vegetative repressor binding site was deleted, its absence would result in derepression of the reporter gene during vegetative growth. Reporter constructs are named on the basis of their 5' terminal position. For example, SPR1/731 corresponds to a reporter containing *SPR1* sequences from -731 to +1 where the *SPR1* ATG is joined in-frame to *lacZ* reporter gene.

2.2b Time Course Analysis of SPR1/762

The *SPR1* promoter (-762 to +1) was fused in-frame to the *lacZ* gene to form translational reporter SPR1/762. To determine that SPR1/762 was regulated in a sporulation-specific manner, β-galactosidase activity was monitored in strain GKY5 at three hour intervals

Figure 11: Alignment of Upstream Activation Sequences of Sporulation-Specific Genes. *SPR1* promoter sequences with 10/15 matches to the UAS of the middle sporulation-specific gene *SPS4* are located between -252 and -236 in the reverse orientation. *SPR1* sequences with 12/17 matches to the MSE of the late gene *SPR2,* are located between -293 and -279 in the forward orientation (8/9 matches to the core of the MSE). Sequences with matches to MSE in other sporulation-specific genes are also indicated. Nucleotides without matches to the consensus MSE are shown in lower case. *SMK1,* encodes a sporulation-specific MAP kinase; *DIT2,* encodes a protein involved in dityrosine biosynthesis. Searches were done using Genepro v 4.0 software. Arrows represent orientation of the sequence under consideration.

SPR2	MSE	(Upper)	→	TTTTTTTTTTTTTGTGGCTACG	-268 to -247
		(Lower)	→	TTTTTGTGTCCCATGGC	-246 to -230
SPS4	UAS		←	GGTTTAGTATCCTTTTTTTGTtGgTTCCTG	-142 to -158
SPR1			→	CTTATTTTTGgcGgT	-293 to -279
SPR1			←	TTTTaGTGTCGTTTGG	-252 to -236
SMK1			→	TAATTTGTGACACTTGA	-82 to -67
DIT2				TTTTGcGACGC	
				Consensus MSE TTTTGTGNC	

throughout sporulation (Fig. 12). No induction was observed until six hours in sporulation medium; peak induction was reached at twelve hours in sporulation medium. Since expression of this reporter completely paralleled the reported abundance of the *SPR1* mRNA (Holaway et al., 1985), we tentatively concluded that the region from -762 to +1 contained the minimum sequences needed for proper temporal regulation. Based on this maximum induction all subsequent *SPR1* reporter assays were performed 12 hours after shifting to sporulation medium.

2.2c *SPR1-lacZ* 5' Nested Deletions

A series of 5' nested deletions of the *SPR1-lacZ* constructs were made by joining portions of the *SPR1* 5' regulatory region and ATG in-frame to the *lacZ* reporter gene. β-galactosidase activity was monitored in both vegetative and sporulating cells of strain GKY5. *SPR1* canonical TATA elements are located at -441, -471 and -565. Reporter SPR1/524 lacks one TATA element while reporter SPR1/329 lacks all three canonical TATA elements. A gradual decline in sporulation-specific β-galactosidase levels was observed in the nested deletion series (Fig. 13). A 71% reduction in sporulation-specific induction was observed for constructs from SPR1/762 to SPR1/329. In addition, a 90% reduction in induction was observed from SPR1/762 to SPR1/268. This suggested that activator binding sites were being removed with progressive deletions. A 62% reduction in sporulation-specific activity was observed when sequences from -329 to -268 were deleted (comparing SPR1/329 and SPR1/268), indicating that these sequences may be necessary for proper induction of *SPR1*. Sequences from -268 to +1 are sufficient to confer sporulation-specific regulation to *SPR1*. The latter finding is evident, as SPR1/268 maintains wild type levels of reporter gene expression during vegetative growth and a 77-fold

Figure 12: *SPR1* Time Course. An *SPR1* reporter SPR1/762 (-762 to +1) transformant was assayed at 3 hour intervals for 24 hours after transfer to sporulation medium. β-galactosidase activity, in Miller units/mg protein was plotted against time in hours. Each point is a mean value of three measurements. Error bars indicate the standard deviation.

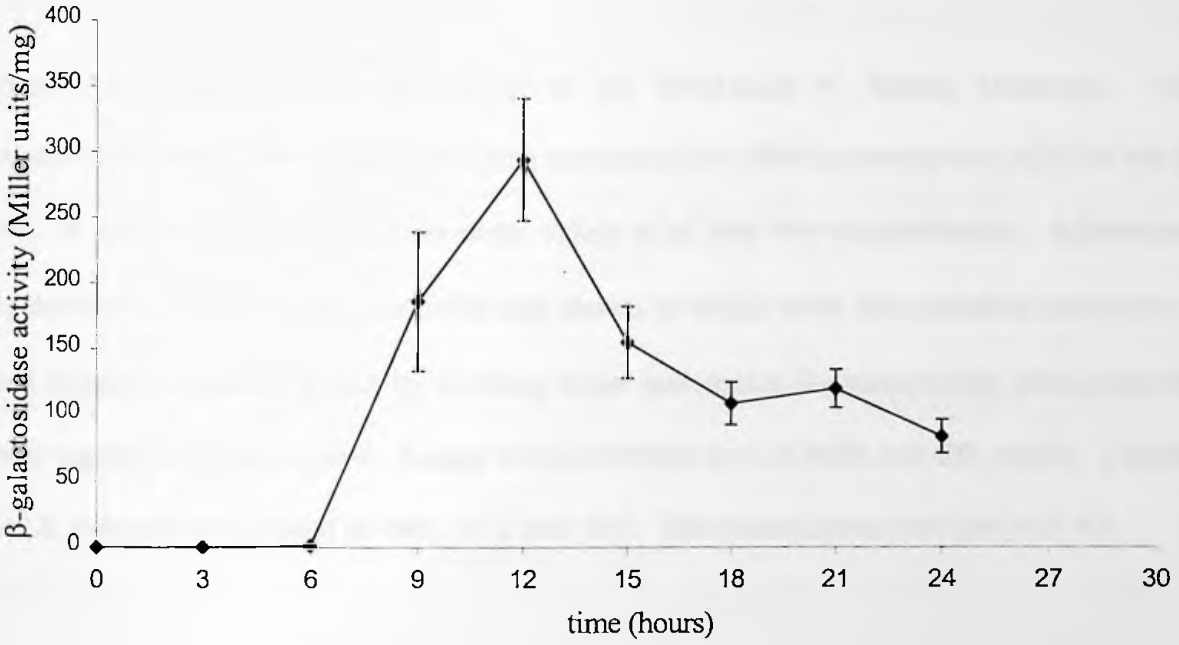
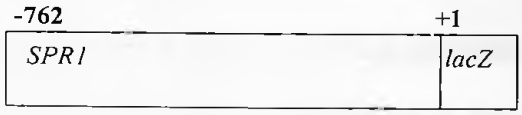
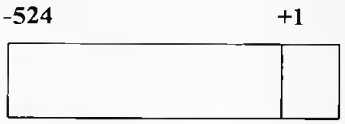
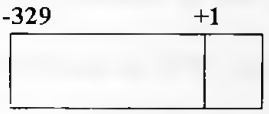
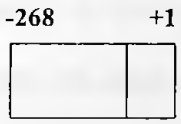
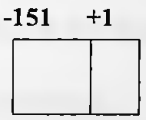
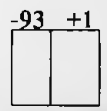

β-galactosidase activity (Miller units/mg)
400
350
300
250
200
150
100
50
0
0
3
6
9
12
15
18
21
24
27
30
time (hours)

Figure 13: Reporter Gene Expression of the *SPR1-lacZ* 5' Nested Deletions. These translational fusions were made by joining portions of the *SPR1* promoter and ATG to the *lacZ* gene. β-galactosidase activities are mean values of at least two measurements. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations (±). Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement. Assays were performed in both PSP_2 and SP_2 media. Canonical TATA elements are located at -441, -471 and -565. The transcription start site is at -65.

		β-galactosidase levels (Miller units/mg)			
		PSP_2	SP_2	%Spo.	Fold
SPR1/762		0.08 ± 0.01	265.5 ± 39.7	50%	3319
SPR1/731		0.09 ± 0.04	160.5 ± 67.7	47	1783
SPR1/524		0.6 ± 0.2	125.5 ± 96.1	47%	209
SPR1/329		0.3 ± 0.1	76.0 ± 24.9	56%	253
SPR1/268		0.4 ± 0.06	28.8 ± 19.4	48%	77
SPR1/151		0.7 ± 0.1	1.3 ± 0.1	48	1.8
SPR1/93		0.9 ± 0.2	0.6 ± 0.06	43%	0.7

induction during sporulation.

SPR1 is controlled by a TATA-less promoter because SPR1/329, which lacks all three canonical TATA elements, is capable of conferring both basal and sporulation-specific regulation of the reporter gene. Reporters SPR1/151 and SPR1/93 were expressed at very low levels during vegetative growth and during sporulation. The low levels of activity observed with SPR1/151 and SPR1/93 could be attributed to two alternative mechanisms: (1) these reporters lack a basal promoter element, or (2) sporulation-specific activation sequences have been deleted. A functional test was designed to distinguish between these possibilities and map the basal promoter of the *SPR1* gene.

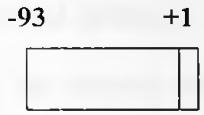
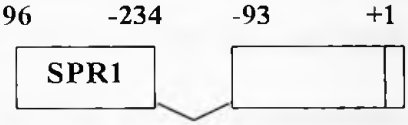
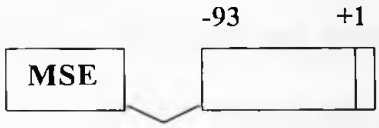
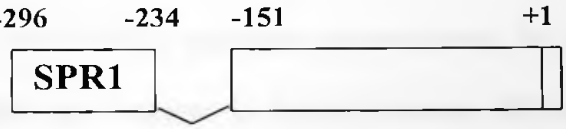
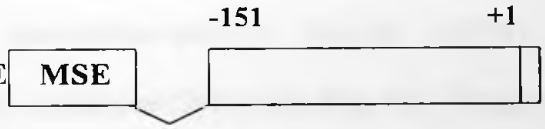
2.2d Mapping the *SPR1* Promoter

In order to map the basal promoter of the *SPR1* gene, a strong UAS, the *SPR2* MSE (Riggs, 1994), was introduced upstream of *SPR1* promoter sequences from -151 to +1 and -93 to +1 to form reporters SPR1/151-MSE and SPR1/93-MSE, respectively. The SPR1/151-MSE reporter was induced during sporulation at levels comparable to *SPR2*/R1 constructs during sporulation (Fig.14). In contrast, SPR1/93-MSE was not induced during sporulation. These findings indicate that sequences from -93 to +1 are not sufficient for basal promoter function, that sequences from -151 to +1 are sufficient for basal promoter function , and that some portion of -151 to -93 is necessary for promoter function.

2.2e Mapping the Activation Sequences of the *SPR1* with Internal Deletions

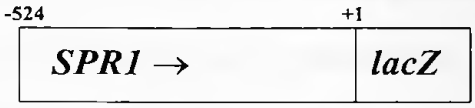
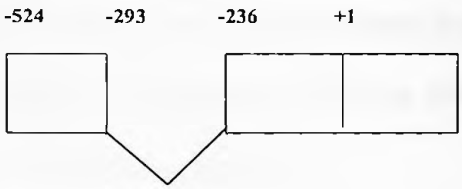
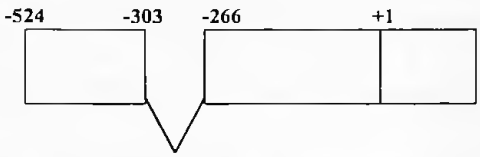
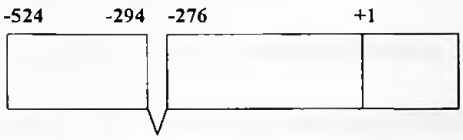
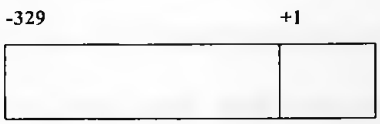
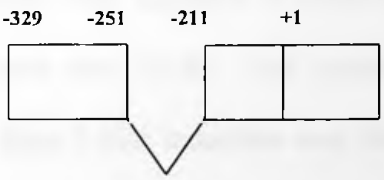
As stated above, searches of the 5' regulatory region of *SPR1* for known *S. cerevisiae*

Figure 14: *SPR1* Promoter is Located Between +1 and -151. SPR1/93-64 and SPR1/151-64 contain a 64 bp *SPR1* sequence joined to the *SPR1* constructs SPR1/93 and SPR1/151; SPR1/93-MSE and SPR1/151-MSE contain a *SPR2* MSE (lower element) joined to the same *SPR1* constructs. These reporters were assayed at 0 hours and 12 hours in PSP_2 and SP_2 media, respectively. ND, <0.01 Miller units of β-galactosidase activity. The β-galactosidase measurements shown are mean values of three or more assays. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations (±). Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement.

		β-galactosidase activity (Miller units/mg protein)			
		PSP_2	SP_2	%Spo.	Fold
SPR1/93		0.9 ± 0.2	0.6 ± 0.06	43%	0.7
SPR1/93-64		0.1 ± 0.01	0.8 ± 0.4	51%	0.13
SPR1/93-MSE		ND	5.3 ± 1.6	58%	–
SPR1/151		0.7 ± 0.1	1.3 ± 0.1	48%	1.8
SPR1/151-64		ND	ND	50%	–
SPR1/151-MSE		ND	136 ± 58.4	59%	–

activation sequences revealed two weak matches. Sequences from -293 to -279 had 10/15 matches to the UAS of the middle gene *SPS4* and sequences from -252 to -236 had 12/17 matches to the MSE of the late *SPR2* gene. Based on these observations and data from nested deletion series (Fig. 13), a series of internal deletions were made within *SPR1-lacZ* reporter SPR1/524 (Fig. 15). Reporters carrying the internal deletions were assayed in both vegetative cells and 12 hour sporulating cells. A 57 bp deletion from -293 to -236 (in reporter SPR1/293del236) deleted both the UAS-like and MSE-like sequences. This deletion led to a 98% reduction of β-galactosidase activity in sporulating cells compared to the parental construct SPR1/524. In order to determine if this reduction was due to both elements or either one separately, less extensive deletions were made and assayed. A 37 bp deletion extending from -303 to -266 in reporter SPR1/303del266 deleted only the UAS-like element. This deletion construct led to a 90% reduction in activity during sporulation; this represents a 20-fold induction compared to 209-fold by the parental construct. A smaller 20 bp deletion from -294 to -276 in sequence SPR1/294del276, also removed the UAS-like sequence and gave a 95-fold induction. Taken together, these deletions suggest that the 37 bp sequence containing the UAS-like element and flanking sequences are necessary for sporulation-specific induction of *SPR1*. Since the MSE-like element is located between -252 and -236, a 40 bp deletion from -251 to -211 was made within reporter SPR1/329 to generate reporter SPR1/251del211. A 99% reduction in sporulation-specific reporter activity was observed comparing reporter SPR1/251del211 to reporter SPR1/329 (Fig 15). These results suggest that sequences from –251 to -211 are also necessary for sporulation-specific expression and induction of *SPR1*. All of the above data suggest that both the UAS-like and MSE-like sequences are necessary for sporulation-specific induction of the *SPR1* gene.

Figure 15: Reporter Expression of *SPR1-lacZ* Internal Deletions. Reporters SPR1/293del236, SPR1/303del266 and SPR1/294del276 were made within translational fusion SPR1/524. Reporter SPR1/251del211 was made within fusion SPR1/329. β-galactosidase activities shown are mean values of three or more assays. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations. Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement.

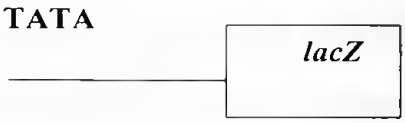
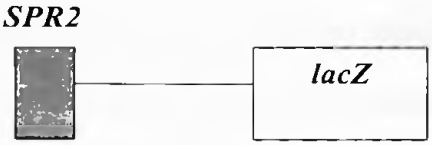
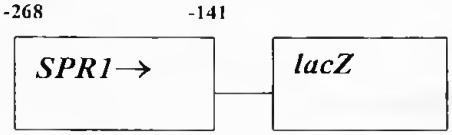
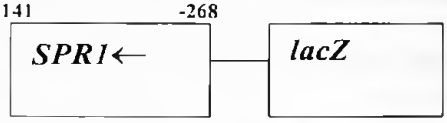
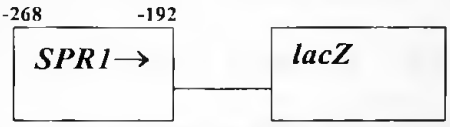
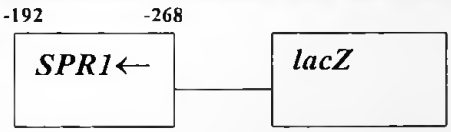
		β-galactosidase levels (Miller units/mg protein)			
		PSP_2	SP_2	%Spo.	Fold
SPR1/524		0.6 ± 0.2	125.5 ± 96.1	47%	209
SPR1/293del236		0.02 ± 0.007	2.78 ± 0.17	35%	154
SPR1/303del266		0.6 ± 0.2	12.0 ± 6.3	28%	20
SPR1/294del276		0.5 ± 0.2	48.9 ± 8.2	38%	95
SPR1/329		0.3 ± 0.2	76.0 ± 24.9	56%	253
SPR1/251del211		0.2 ± 0.05	0.7 ± 0.2	32%	4

To determine if the MSE-like and UAS-like sequences combined are sufficient for sporulation-specific regulation of *SPR1*, sequences from -296 to -234 were joined to the basal promoter reporters SPR1/151 and SPR1/93 to generate SPR1/151-64 and SPR1/93-64 (Fig. 14). Neither construct was induced during sporulation. Reporter SPR1/93-64 does not contain sequences sufficient to confer basal promoter activity as observed with reporter SPR1/93-MSE (Fig. 14) and therefore was not expected to induce β-galactosidase activity. Taken together data from the internal deletions suggest that the 64 bp *SPR1* sequence is necessary but not sufficient to confer sporulation-specific regulation.

2.2f Analysis of *SPR1-CYC1-lacZ* Constructs

To test if the MSE-like elements were functional UASs, transcriptional fusions containing this element were made by inserting *SPR1* promoter fragments -268 to -141 and -268 to -192 into the enhancerless *CYC1-lacZ* reporter in both forward and reverse orientations to generate the reporters SPR1/268-141F, SPR1/268-141R, SPR1/268-192F and SPR1/268-192R (Fig. 16). These fragments were expected to confer sporulation-specific induction independent of orientation if they were true UASs. For reporters SPR1/268-141F, SPR1/268-192F and SPR1/268-192R, a less than 2-fold induction was observed during sporulation and for reporter SPR1/268-141R an approximately 4-fold induction was observed. Surprisingly, all the constructs except SPR1/268-141F were expressed at relatively high levels in vegetative cells in comparison to 312ΔX and SPR2/R1F (*SPR2*-MSE). These findings show that sequences from -268 to -141 are not sufficient to confer sporulation-specific regulation, but can confer constitutive activation of the reporter gene because these reporters are expressed during

Figure 16: Expression of *SPR1-CYC1-lacZ* Transcriptional Fusions. Expression levels were monitored in PSP_2 and SP_2 media. β-galactosidase activities shown are mean values of three or more assays. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations as described. Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement. 312ΔX is the enhancerless *CYC1-lacZ* reporter. SPR2/R1F is an *SPR2*-MSE-*CYC1-lacZ* construct. F and R indicate forward and reverse orientation, respectively.

		β-galactosidase levels (Miller units/mg protein)			
		PSP_2	SP_2	%Spo.	Fold
312ΔX		4.3.± 0.9	2.6 ± 0.4	69%	0.6
SPR2/R1F		5.0 ± 1.0	109.0 ± 14.8	62%	22
SPR1/268-141F		6.3 ± 0.5	9.7 ± 1.5	34%	1.5
SPR1/268-141R		20.7 ± 6.7	75.5 ± 34.9	49%	3.6
SPR1/268-192F		17.6 ± 2. 3	28.2 ± 10.7	42%	1.6
SPR1/268-192R		48.5 ± 7.5	65.5 ± 29.6	45%	1.3
SPR1/296 234F		9.21 ± 2.0	3.1 ± 0.7	50%	0.3

vegetative growth. These data suggest that the SPR1/268-141 and SPR1/268-192 reporters lack a repressor binding site.

Since deletion of the above sequences (-251 to -211, in SPR1/251del211) exhibited a 99% reduction in activity in sporulating cells (Fig. 15), a smaller construct, SPR1/296-234F which contains both the MSE-like and UAS-like sequences (-296 to -234), was tested (Fig. 16). Expression of this construct was inhibited during sporulation; as a 0.3-fold induction was observed, consistent with a repressor binding site within this reporter.

2.2g Regulation of *SPR1* by Sporulation-Specific Regulators

The dependence of *SPR1* expression on sporulation-specific regulators *IME2*, *UME6* and *NDT80* was tested by transforming mutant strains of the three regulators with two *SPR1* reporters, SPR1/731 and SPR1/524. Expression of both constructs was reduced dramatically during sporulation in all three mutant strains. Low levels of β-galactosidase activity were detected in the *ndt80* mutants (Table 4). All three mutant strains showed less than 5% sporulation efficiency compared to 47% of the wild type strain. The failure of the reporters to be induced during sporulation indicates that all three proteins are positive regulators of the *SPR1* gene

2.2h Sporulation-Specific Protein-DNA Interactions

Three different *SPR1* probes SPR1/GSS294 (-294 to -234, 60 bp, containing the UAS-like and MSE-like elements), SPR1/GSS293 (-293 to -251, 42 bp, containing the UAS-like

Table 4: The Role of *IME2, UME6* and *NDT80* in the Regulation of the Late Gene *SPR1*. *ime2* (AMP245), *ume6* (RSY239) and *ndt80* (YSC508) were transformed with constructs SPR1/731 and SPR1/524. β-galactosidase activity was assayed at both 0 hours and 12 hours in PSP_2 and SP_2 media, respectively. Expression of β-galactosidase reporter was quantified and shown as Miller units with standard deviations as described. Fold induction was calculated by dividing mean sporulation β-galactosidase measurement by mean vegetative measurement. ND, β-galactosidase activity <0.01 Miller units. % spo indicates sporulation efficiency. β-galactosidase activities shown are mean values of three or more assays.

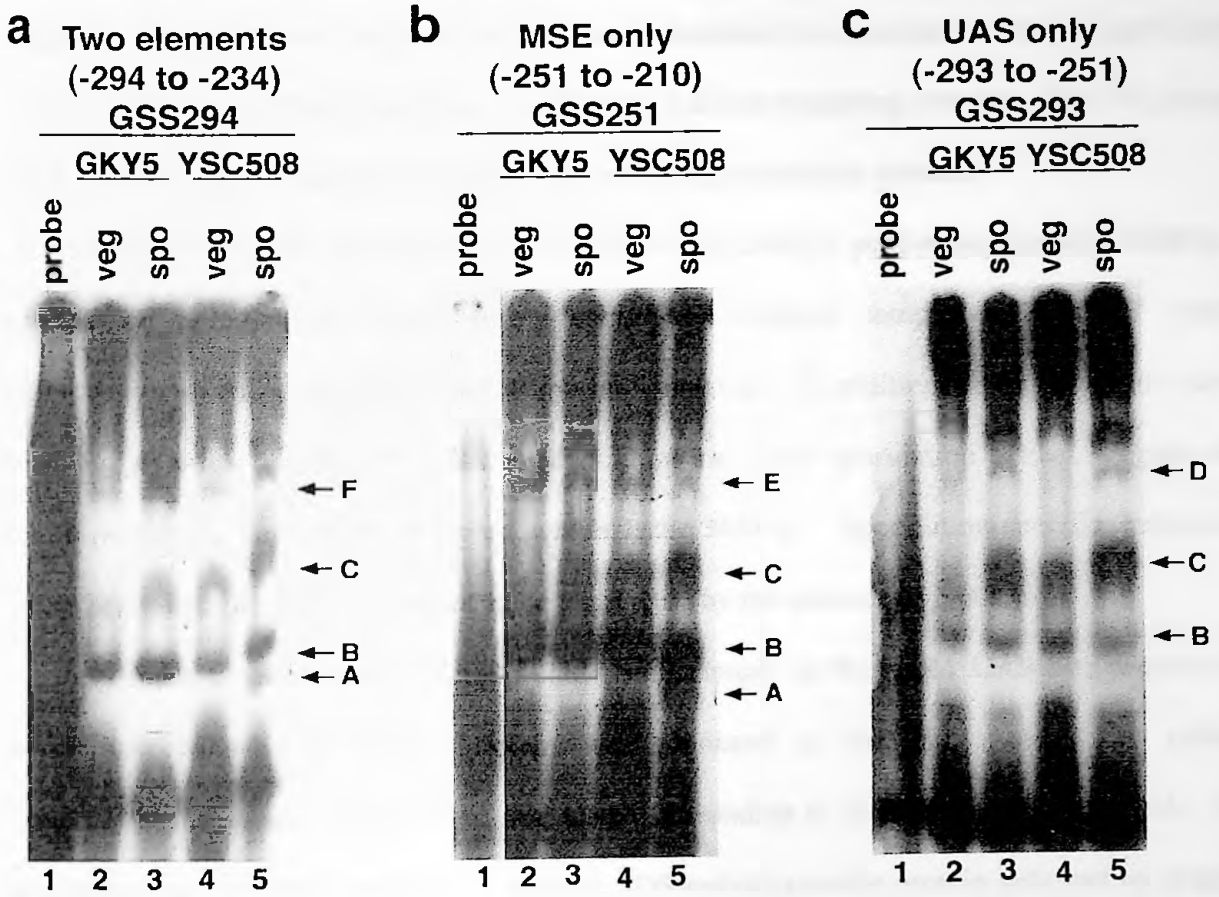
	SPR1/731				SPR1/524			
Strain	**PSP_2**	**SP_2**	**%SPO.**	**Fold**	**PSP_2**	**SP_2**	**%SPO.**	**Fold**
Wild type	**0.09 ± 0.04**	**160.5 ± 67.7**	**47%**	**1783**	**0.6 ± 0.2**	**125.5 ± 96.1**	**47%**	**209**
ime2	**ND**	**ND**	**0%**	**–**	**ND**	**ND**	**5%**	**–**
ume6	**ND**	**0.5 ± .09**	**1%**	**–**	**ND**	**ND**	**1%**	**–**
ndt80	**0.08 ± 0.01**	**0.48 ± 0.18**	**0%**	**6**	**0.07 ± 0.05**	**0.54 ± 0.07**	**0%**	**8**

element) and SPR1/GSS251 (-251 to -210, 41 bp, containing the MSE-like element) were used to determine if these promoter fragments bind either vegetative or sporulation-specific proteins. Crude vegetative and sporulation protein extracts were prepared from strains GKY5 and YSC508 (*ndt80* mutant strain). A series of complexes was observed with both wild type and ndt80 mutant strains. With probe SPR1/GSS294 (both elements) one of the fastest migrating complexes was present in equal amounts when vegetative and sporulation extracts from either the wild type or *ndt80* mutant strains were used (Fig. 17a, band B). Other slower migrating complexes (C and F) were present in greater abundance in reactions containing wild type sporulation extracts (Fig. 17a, compare lanes 2 and 3). Complex F is diminished in the *ndt80* mutant extract reaction relative to the wild type (Fig. 17a, compare lanes 3 and 5), while complex C is increased in intensity in the *ndt80* mutant reaction relative to wild type (Fig. 17a, compare lanes 3 and 5). Complex C is also present in the mutant vegetative lane (lane 4). Finally, a novel fast migrating complex (A) is formed only in binding reactions containing the sporulating *ndt80* mutant protein extract (Fig. 17a, lane 5).

With probe SPR1/GSS251 (MSE-like element) probe, one of the fastest migrating complexes was present in equal amounts when vegetative and sporulation extracts from either strain was used (Fig. 17b, complex B). A slow migrating complex was present in greater abundance with sporulation extracts of both the wild type and *ndt80* mutant strains (complex C). A slower migrating complex (Fig. 17b, complex E) was present with both wild type and *ndt80* mutant extracts, but was of relatively greater intensity with the wild type sporulation extract. Finally, a novel complex similar to the complex in Fig. 17a is formed only in binding reactions containing the ndt80 mutant protein extract (Fig. 17b, complex A).

In EMSAs with the SPR1/GSS293 (UAS-like element) probe, the fastest

Figure 17: EMSAs with *SPR1* Promoter Sequences and Crude Protein Extracts. **17a**) probe SPR1/GSS294; **17b**) probe SPR1/GSS251; **17c**) SPR1/GSS293. The probe alone was run in lanes 1a, 1b and 1c. The binding reactions contained probe and 25 μg of vegetative GKY5 extract (2a, 2b and 2c), 20 μg of GKY5 sporulation extract (3a, 3b and 3c), 25 μg of YSC508 (*ndt80* mutant) vegetative extract (4a, 4b and 4c), or 25 μg of YSC508 (*ndt80* mutant) sporulation extract (5a, 5b and 5c), respectively. A, *ndt80* mutant sporulation-specific complex (17a and 17b, lane 5); B, complexes in 17a, 17b and 17c which formed with vegetative and sporulation extracts of both wild type and *ndt80* mutant strains; C, complexes observed with all extracts except vegetative wild type (17a, 17b and 17c, lanes 3-5); D, sporulation-specific complex (17c only, lanes 3 and 5); E, complexes observed with vegetative and sporulation extracts (17b only, lanes 2-5); and F, complex observed with wild type and *ndt80* mutant sporulation extracts with more intensity than with their respective vegetative extracts.

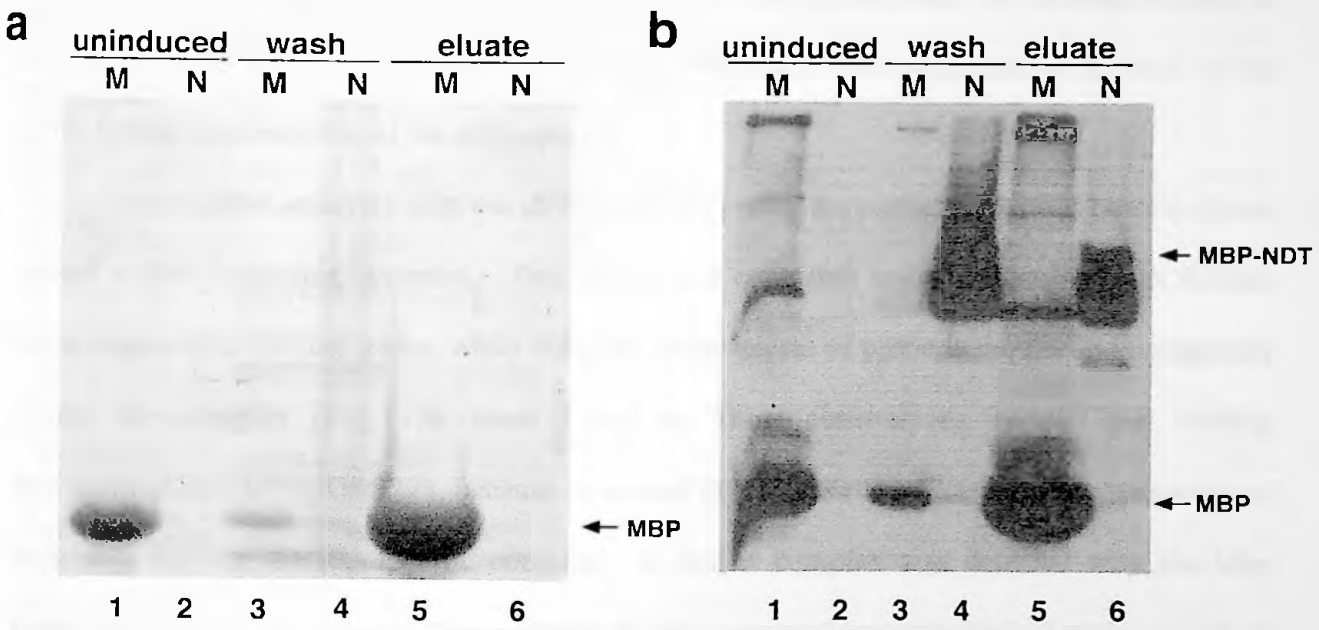

a
Two elements
(-294 to -234)
GSS294
GKY5 YSC508
probe
veg
spo
veg
spo
F
C
B
A
1 2 3 4 5
b
MSE only
(-251 to -210)
GSS251
GKY5 YSC508
probe
veg
spo
veg
spo
E
C
B
A
1 2 3 4 5
c
UAS only
(-293 to -251)
GSS293
GKY5 YSC508
probe
veg
spo
veg
spo
D
C
B
1 2 3 4 5

migrating complex (Fig. 17c, complex B) was present in equal amounts when vegetative and sporulation extracts from either the wild type or ndt80 mutant strains were used. A slow migrating complex (band C) was present in greater abundance in reactions containing sporulation extracts (Fig 17c, compare lanes 2 and 3). Finally, a slower migrating complex (Fig 17c, lanes 3 and 5, band D) was present only in reactions containing sporulation extracts.

In summary, the EMSAs with the GSS294 and GSS251 probes suggest that Ndt80p is required for complex formation during sporulation because complexes E and F appear diminished in reactions containing *ndt80* sporulation extract. In addition, the presence of a faster migrating complex (band A) in reactions containing the *ndt80* sporulation extract suggests that this might be an incomplete complex which lacks Ndt80p. Sporulation-specific complexes which formed on the GSS293 appear to be unaffected by the absence of Ndt80p.

In order to determine if Ndt80p could bind directly to the SPR1 UAS-like sequence, a recombinant Mbp-Ndt80 fusion protein was produced in bacteria, purified by affinity chromatography on amylose agarose, and tested for binding to SPR1/GSS293 by EMSA. The recombinant protein was analyzed by staining SDS-polyacrylamide protein gels and by western blotting to determine the extent of purification. On a Coomassie-stained gel, fewer bands of greater intensity were observed with the Ndt80p eluate compared to Mbp-Ndt80 wash fraction (Fig. 18a, lanes 4 and 6, respectively). A band in the Ndt80p wash fraction and eluate (Fig. 18b, lanes 4 and 6) comigrated with the 116 kD molecular weight marker (data not shown). This was consistent with the expected (calculated) molecular weight of the Mbp-Ndt80p fusion protein which is 114 kD. (Mbp is 42.6 kD and Ndt80p is 71.4 kD). An identical gel immunoblotted with anti-Mbp antibody revealed multiple bands in the fusion protein wash fraction and eluate

Figure 18: Analysis of Affinity Purified Mbp-Ndt80p. **a)** Coomassie stained protein gel. **b)** Western blot immunoblotted with anti-Mbp antiserum. 25 μg of protein was electrophoresed on a SDS-polyacrylamide gel. The samples were uninduced (-IPTG) crude extract (lanes 1a, 1b, 2a and 2b), induced (+IPTG) wash (3a, 3b, 4a and 4b), and partially purified protein inducted (+IPTG) eluate (lanes 5a, 5b, 6a and 6b), respectively. M, Mbp expression vector lysate. N, Mbp-Ndt80 expression vector lysate.


a
uninduced
wash
eluate
M N M N M N
MBP
1 2 3 4 5 6
b
uninduced
wash
eluate
M N M N M N
MBP-NDT
MBP
1 2 3 4 5 6

(Fig. 18b, lanes 4 and 6). The 116 kD band was bound the anti-Mbp antibody. The bands migrating faster than Mbp-Ndt80p may be either bacterial contaminants or proteolytic cleavage products. Proteolytic cleavage cannot be completely ruled out even though, the bacterial host strain was protease deficient. The low molecular weight bands bound to anti-Mbp antibody could be endogenous Mbp of the bacterial host strain The strong signals observed with Mbp antiserum in lanes 4 and 6 (Fig. 18b) suggest that a large amount of Mbp-Ndt80p fusion protein was present in the wash. This can be attributed to low binding efficiency of the Mbp-Ndt80p to the amylose column (Fig. 18b, lane 4). This low efficiency in binding may be a result of the native folded conformation of the Mbp protein.

Upon EMSA analysis with the SPR1/GSS293 probe, the partially purified Ndt80p eluate formed a slow migrating complex. This complex is competed out considerably with 50-fold molar excess of unlabeled probe, while 100-fold molar excess of probe is required to completely remove the complex (Fig. 19a, lanes 3 and 4). These observations suggest that Ndt80p specifically binds SPR1/GSS293, because unlabeled SPR1/GSS293 oligonucleotide is capable of competing out the Ndt80p-specific complex. A similar complex was detected with the Mbp eluate alone (Fig. 19a, lane 6). This complex is also competed out with 50-fold molar excess of unlabeled probe (lane 7), even though the specificity of this complex is not known.

To further support these findings, competition assays were performed with a MSE mutant (GCCAGTAACAC, Mut C) sequence. These EMS competition assays were compared to competition with the true MSE. The Ndt80p specific complexes were competed out with 100-fold molar excess of unlabeled MSE probe SPR2/GSS259 (Fig. 19b, lanes 6 and 7); two hundred fold molar excess of the 11 bp unlabeled mutant (Mut C) sequence was incapable of competing out this complex (Fig. 19b, lanes 3-5). This suggested that an MSE-like element within

Figure 19a: EMSAs with Affinity Purified Mbp-Ndt80p. The probe alone was run in lane 1. The binding reactions contained probe and 2.8 μg of Mbp-Ndt80p (lanes 2-5), or 2.8 μg of Mbp (lanes 6-9). Some reactions also contained molar excesses of unlabeled SPR1/GSS293 as indicated in the top panel (lanes 3-5 and 7-9). Comp, Competitor. Arrow indicates the position of the Ndt80p-specific complex.

Figures 19b: Competition EMSAs of SPR1/GSS293/Ndt80p Complex by Mut C and the SPR2 MSE. The probe alone was run in lane 1. The binding reactions contained probe and 2.8 μg of Mbp-Ndt80p (lanes 2-7). Some reactions also contained molar excesses of unlabeled Mut C competitor (lanes 3-5) or unlabeled SPR2/GSS259 (lanes 6-7) competitor as indicated on the top panel. Comp, Competitor. Arrow indicates the position of the Ndt80p-specific complex.

a
GSS293 probe
(UAS-like)
MBP-NDT80
GSS293
50x comp
100x comp
200x comp
MBP
GSS293
50x comp
100x comp
200x comp
Ndt80p-
GSS293
1 2 3 4 5 6 7 8 9
b
GSS293 probe
(UAS-like)
MBP-NDT80 eluate
Mut C
50x comp
100x comp
200x comp
GSS259
100x comp
200x comp
Ndt80p-
GSS293
1 2 3 4 5 6 7

SPR1/GSS293 bound Ndt80p and that specific nucleotides were necessary to facilitate the interaction between Ndt80p and MSE sequences (Fig.20b, lanes 3-5).

3.0 DISCUSSION

3.1 The *SPR2* gene is Regulated by MSEs

The induction of the late sporulation-specific gene *SPR2* is controlled by a sequence from -259 to -230 that contains two consensus MSEs. Each individual MSE is sufficient to confer sporulation-specific regulation of the reporter gene independent of orientation. Therefore, each MSE is an UAS. The composite MSE is required to confer maximal induction of *SPR2*, since a 5-fold greater induction is observed with the composite MSEs compared to the individual MSEs (Table 2). This suggests that Ndt80p binds the *SPR2* MSE as dimers or as multiple dimers, and that the dimer is required for synergistic induction. The GC rich sequences at the 3' end of the lower MSE are necessary for complete induction of the *SPR2* gene, because replacement of these bases with AT rich sequences leads to reduction in sporulation-specific induction (Table 2). Hepworth et al. (1995) also showed that mutations of the GC rich sequences of the *SPS4* MSE lead to a large reduction in the level of expression.

The *SPR2* gene is positively regulated by two early regulators, *IME2* and *UME6,* and by a middle regulator *NDT80*. The MSE sequences of the *SPR2* gene interact directly with Ndt80p as demonstrated by EMSAs (Fig. 10a). Conserved bases within the MSE (TTTTTGTG) are necessary to mediate interactions between Ndt80p and MSE sequences, as a mutant MSE (CGGTCATTGTG, Mut C, bases with mismatches to the consensus MSE underlined) is incapable of competing out the Ndt80p-wild type MSE complex at a 200-fold molar excess concentration (Fig. 10b). Consistent with this interpretation, the Mut C sequence was incapable of disrupting the Ndt80p-MSE complex between *SPS4* MSE and recombinant Ndt80p in competition EMSAs conducted by Chu and Herskowitz (1998).

Ndt80p, a middle sporulation-specific regulator, is believed to confer regulation through the MSE of middle and late genes. MSE sites are present in both middle sporulation-specific genes (*SPS1* and *SPS4*) and other sporulation genes (*CLB1-6*, *DIT2* and *SGA1*) (Chu and Herskowitz, 1998; Hepworth et al., 1995). Direct binding of the recombinant Ndt80p to the *SPS4* MSE was demonstrated by EMSAs (Chu and Herskowitz, 1998). Three pieces of evidence from our studies suggest that Ndt80p regulates *SPR2* expression directly by binding to the 5' regulatory region. (1) Null mutants of *ndt80* fail to express *SPR2-CYC1-lacZ* reporters (Table 3). (2) The 5' regulatory region of *SPR2* contains two consensus MSE sequences that confer sporulation-specific regulation independent of orientation (Table 2). (3) An *SPR2* MSE binds Ndt80p in EMSAs and this binding is competed out by unlabeled oligonucleotides. Furthermore, a mutant MSE is incapable of competing with the MSE probe (Figure 10). The presence of a large, strong signal was of some concern (Fig. 10a, lane 2). On shorter auoradiographic exposures, at least two shifted bands are visible (data not shown). Since the SPR2/GSS259 probe used in these studies contained both MSEs, the presence of multiple complexes can be explained by binding of Ndt80p to one or both MSEs. An alternate explanation for the strong signal could be that posttranslational modifications or proteolytic cleavage of Ndt80p would lead to Ndt80p molecular weight variants (which could still bind the MSE.

In this study, MSE binding reactions which contained Mbp showed a shifted band. It is unlikely that Mbp itself is binding to the MSE for a number of reasons. First, purified recombinant Mbp has been shown not to bind the MSE under the same conditions used in the study (Chu and Herskowitz, 1998). Second, the intensity of this shifted band is much less than the Mbp-Ndt80 fusion protein band in the original autoradiograph (data not shown). Since densitometric evaluation of SDS-polyacrylamide gel indicated approximately 69% purity (Fig.

18), contaminant bacterial proteins are present in the Mbp preparation and these proteins may be responsible for the shifts.

In EMSAs with GKY5 (wild type) crude extracts, sporulation-specific shifted bands were observed; surprisingly, these bands were present with diminished intensity in reactions performed with YSC508 (*ndt80* mutant; Fig. 9). One possible explanation for similar complexes formed with both wild type and *ndt80* mutant strains could be the participation of a protein with similar molecular weight to Ndt80p in complex formation in the *ndt80* mutant strain. This supports the notion that Ndt80p binds to the MSE directly in wild type cells and participates in gene regulation. Alternatively, the less intense bands observed in *ndt80* mutant strain might be a consequence of fewer complexes forming as a result of the absence of Ndt80p. The latter argument suggests that Ndt80p facilitates complex formation, but may not be through direct DNA binding.

SPR2-lacZ reporters are not induced during sporulation in *ime2*, *ume6* or *ndt80* mutant strains. The exact mechanism of regulation of the *SPR2* gene by the positive regulators *IME2* and *UME6* is unclear. Searches of the 5' regulatory region of *SPR2* revealed three sequence elements with 6/9 matches to the *UME6* binding site, URS1, one of which overlaps the upper MSE. As previously mentioned, Ume6p is a negative regulator when bound to the URS1 site (Strich et al., 1994; Park et al., 1992) and is a positive regulator in the presence of Ime1p (Rubin-Bejerano et al., 1996). These findings and the dual role of *UME6* in the regulation of sporulation-specific genes suggest that Ume6p, along with Ndt80p, might bind the MSEs and regulate the *SPR2* gene. Ime2p is a serine threonine kinase, which is believed to phosphorylate sporulation-specific targets (Kominami et at., 1993). An *IME2* target protein could be the actual *SPR2* regulator. Ndt80p, a delayed early gene, is a possible target of *IME2,* as this protein is

11.2% serine and 4.9% threonine (Xu et al., 1995).

These findings suggest that expression of the *SPR2* gene is a highly controlled process involving interactions between specific cis-acting sequences and transactivators. Ndt80p is a transactivator that has been shown to regulate transcription of middle genes (Chu and Herskowitz, 1998). The MSE sequences have a 12/17 match (Fig. 7) to the NRE (negative regulatory element) of the late sporulation-specific gene *SGA1* (Kihara et al, 1991). Consistent with this limited homology, the *SPR2* MSE does not down-regulate a *CYC1-lacZ* reporter (Riggs, 1994). This suggests that differences in nucleotides between the MSE and NRE or flanking sequence are required for negative regulation. These differences in nucleotide sequence between the MSE and NRE may determine the type of interaction between the protein and the target sequence; in some instances providing binding sites for activators, and in others facilitating interactions with repressors.

3.2 The *SPR1* Gene is Regulated by a Composite Promoter.

The regulation of the late sporulation-specific gene *SPR1* was studied using both genetic and molecular approaches. Since *SPR1* regulation is maintained even after all three canonical TATA elements (located at positions -441, -471 and -565) are deleted (Fig. 13), a mapping study was performed by joining the *SPR2* MSE to *SPR1-lacZ* reporters. This study indicated that *SPR1* sequences from -151 to +1 are sufficient to confer basal promoter activity and that part or all of the sequences between -151 and -93 are necessary for this function (Fig. 14). Two 4/6 matches and other weak matches to the canonical TATA element are found between -151 to +1; these elements could serve as the basal promoter elements.

In addition to providing evidence that *SPR1* is regulated by a TATA-less promoter, the sequential deletions of the *SPR1* promoter region from -762 to -93 showed a gradual decline in sporulation-specific reporter activity. This indicates that several different elements participate in the regulation of *SPR1*. Further analysis of the reporter genes indicated two decreases in sporulation-specific induction between SPR1/329 and SPR1/268, and between SPR1/268 and SPR1/151 (Figure 13). This region from -268 to +1 is the minimum sequence needed to confer sporulation-specific regulation of the *SPR1* gene, that is, to insure low levels of expression during vegetative growth and induction during sporulation; other sequences upstream of -268 are required for full sporulation-specific induction. Searches of the 5' regulatory region of *SPR1* for known *S. cerevisiae* activation sequences revealed two matches: (1) 10/15 match to the UAS sequence of the middle sporulation gene *SPS4* (UAS-like element) and (2) 12/17 match to the MSE of the late sporulation gene *SPR2* (MSE-like element) (Fig. 11).

Internal deletions of the UAS-like or MSE-like sequences within nested *SPR1-lacZ* reporters (SPR1/303del266 and SPR1/251del211) showed dramatic reductions in sporulation-specific reporter gene expression, from 125.5 to 12.5 miller units/mg, and from 76.0 to 0.7 miller units/mg, respectively (Fig. 15). These >90% reductions in sporulation-specific induction suggest that the UAS-like and MSE-like elements are necessary for complete induction.

However, *SPR1-CYC1-lacZ* constructs containing the UAS-like element (-268 to -141 and -268 to -192), tested in both the forward and reverse orientations, suggest that this sequence is not sufficient for sporulation-specific regulation, as all constructs except SPR1/268-141F are derepressed during vegetative growth (Fig. 16). These data also suggest that sequences from -268 to -141 contain a constitutive activation sequence, and that the constitutive element functions more efficiently in the reverse orientation than in the forward orientation.

Alternatively, these findings may also suggest that an essential negative regulatory element was deleted in generating these fusions or that an activation sequence was generated during construction of these reporters. The latter is hard to resolve, as the exact sequence that might confer such activation has not been defined.

In addition, SPR1/296-234, which contains both the UAS-like and MSE-like elements fused to the *CYC1*-lacZ reported, is not sufficient for maximal induction; this 64 bp sequence actually confers a slight repression during sporulation (Fig. 16). Constructs SPR1/151-64 and SPR1/93-64, made by joining a 64 bp sequence containing the UAS-like and MSE-like elements (-296 to -234) to *SPR1* promoter fragments -151 and -93, further support this observation as the 64 bp sequence is not sufficient to confer sporulation-specific induction of the reporter gene (Fig. 14). All of the above observations taken together indicate that sequences from -293 to -234 are necessary but not sufficient for maximal sporulation-specific induction. Additional flanking sequences must be required to maintain complete regulation of the *SPR1* gene.

Reporter gene expression studies performed in *ndt80* mutant strains suggested that Ndt80p was a positive regulator of the *SPR1* gene. Therefore, EMSAs were performed to establish that Ndt80p binds *SPR1* cis-acting sequences during sporulation. A fast migrating complex (band A) was produced in binding reactions containing crude YSC508 (*ndt80*) sporulation extracts and either SPR1/GSS294 (MSE-like and UAS-like sequences) or SPR1/GSS251 (MSE-like sequence) (Fig. 17a and 17b, lane 5). This finding suggests that Ndt80p is a component of the slow migrating complexes (Fig. 17a, band F and Fig. 17b, band E). This observation is further supported by the presence of a stronger signal with wild type extracts compared to a weaker signal with *ndt80* mutant extracts; (Fig. 17a, compare band F in lanes 3 and 5, F; Fig. 17b, compare band E in lanes 3 and 5). These data suggest that complex A is a

partially formed complex due to the loss of Ndt80p in the YSC508 strain, while complexes E and F are completely formed complexes in the presence of Ndt80p from the GKY5 strain. Therefore, we conclude that Ndt80p is an important component of the complex binding to the *SPR1* DNA probe.

Ndt80p is a middle regulator that binds the MSEs of sporulation genes (Chu and Herskowitz, 1998). *SPR1* expression was reduced dramatically in these *ndt80* mutants, and sporulation was incomplete as cells arrest at the end of prophase I. EMSAs with recombinant Ndt80p show that SPR1/GSS293 probe (UAS-like) interacts directly with Ndt80p. This complex is considerably competed out with 50-fold molar excess of unlabeled probe, suggesting that the protein-DNA complex is specific to the UAS-like sequence (Fig. 19a, lanes 2-4, arrow). This interaction is not interrupted by a mutant MSE sequence (Mut C) while it is competed out by 100-fold molar excess of the true MSE (Fig. 19b, lanes 2-5, and lanes 6 and 7). These data suggest that the Ndt80p is capable of binding to both MSE and UAS-like sequences, and further that specific nucleotides are required to mediate this protein-DNA interaction.

A non-specific complex was observed in all binding reactions with crude extracts from wild type and *ndt80* mutant cells (Fig.9, lanes 2-9, NS; Fig.17 a, 17b, 17c, B). This complex is not competed out by unlabeled probe. However, a similar complex was not detected in binding reactions with recombinant protein. These observations might suggest that the nonspecific complex is formed by more abundant nuclear proteins, such as histones present in the crude extracts.

Two early regulators Ime2p and Ume6p act as positive regulators of the *SPR1* gene, as *ime2* and *ume6* mutants dramatically reduce *SPR1* expression and block sporulation. It is unlikely that either gene regulates *SPR1* directly. The absence of URS1 sites (Ume6p binding

sites) in the 5' regulatory region of *SPR1* rule out direct regulation by *UME6*. Ime2p is a kinase (Kominami et al., 1993) and therefore may phosphorylate a potential *SPR1* transactivator protein such as Ndt80p.

In summary, the regulation of two late sporulation-specific genes was studied in our laboratory employing both genetic and molecular approaches. *SPR1* and *SPR2* are considered members of the late class of genes, based on their peak time of expression, but are subjected to highly controlled expression. This classification system is very broad, as time of expression varies depending on the strain under consideration. *SPR1* and *SPR2* may be considered mid-late genes for the following reasons: (1) expression of both genes peaks after eight hours in SP_2 medium, (2) both genes are regulated by the middle regulator, Ndt80p, and (3) *ime2* or *ume6* null mutants strains do not express *SPR2* or *SPR1*. *SPR1* encodes an exo-1,3-β-glucanase believed to be involved in spore wall maturation while, the function of *SPR2* is unknown. Because *SPR1* and *SPR2* have characteristics of both middle and late genes they may be classified as mid-late genes. The 5' regulatory regions of *SPR1* and *SPR2* have no homology. But both genes are positively regulated by two early regulators, *IME2* and *UME6,* and by a middle regulator, *NDT80*. Analysis of the regulatory sequences of *SPR2* reveals two consensus MSEs in tandem from -260 to -230, while *SPR1* has no consensus MSEs or other UASs. The MSEs are sufficient to confer full sporulation-specific regulation of *SPR2*, while more than one element in the SPR1 promoter (sequences from -329 to +1) is necessary to confer maximal sporulation-specific regulation of *SPR1*. *SPR2* and other sporulation-specific genes (*SPR3* and *SPS4*) are classic Ndt80p-dependent genes, while *SPR1* regulation is unique. Expression of *SPR1* is dependent on Ndt80p, but is not mediated through a consensus MSE. Detailed studies of the *SPR1* promoter region provide strong evidence to suggest that the *SPR1* gene is regulated by a composite TATA-

less promoter. These studies reveal some interesting conclusions that may apply to any eukaryotic system. Transcriptional regulation of genes participating in the same differentiation pathway is not necessarily determined by the same factors. It requires highly controlled interplay between various protein complexes and their target sequences to confer maximal regulation.

4.0 MATERIALS AND METHODS

4.1 *S. cerevisiae* Strains Used in this Study

The genotypes of the strains used in this study are listed in Table 5. These strains were obtained from Aaron Mitchell and Randy Strich. Some strains were obtained as diploid a/α cells, while some diploid strains were obtained by mating opposite mating types **a** and α. The diploid GKY5 strain was used as the wild type recipient strain for reporter plasmids in this study. Haploid *ume6* mutant strains RSY237 and RSY238 were mated to obtain a diploid *ume6* mutant. This diploid *ume6* mutant strain and other diploid mutant strains, namely AMP245 (*ime2*) and YSC508 (*ndt80*), were used to perform reporter assays as indicated. Competent *S. cerevisiae* cells were prepared by the lithium acetate method, making a minor modification to the protocol described by Ausubel et al. (1994).

4.1a *S. cerevisiae* Culture Conditions and β-galactosidase Assays

Crude extracts of yeast cultures for β-galactosidase assays were prepared according to the following protocol. A single colony of a yeast transformed with reporter plasmid was picked from uracil selective medium, inoculated into 1.5 ml of minimal glucose medium (Table 6) and incubated at 30°C with shaking for 12 hours. Liquid YEPD medium (1 ml) was added to the culture which was shaken for an additional 10 hours at 30°C. This culture was then used to inoculate 60 ml of presporulation medium (PSP_2; Table 6). PSP_2 cultures were shaken at 30°C until a cell density of 2-4 × 10^7 cells/ml was reached. Cells were then washed twice with an equal volume of sterile H_2O and resuspended in 1.25 ml of sterile H_2O.

Table 5: ***S. cerevisiae*** **Strains Used in this Study**

GKY5	MAT**a**/ MATα, his3/ his3, leu2-3,112/ leu 2-3,112, ura 3-52/ ura 3-52
AMP245	MAT**a**/ MATα, ime2-2::LEU2, ura3, leu2::hisG, trp1::hisG, lys2, ho::LYS2
RSY238	MAT**a**, his3^{-}, leu2, lys1, lys2, trp1, ume 6-2, ura3
RSY237	MATα, his4, leu2, lys1, trp1,ume6-2,ura3
YSC508	MAT**a**/ MATα ndt80::LEU/ ndt80::LEU, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, psi-, GAL+

Table 6: Media and Growth Conditions for *S. cerevisiae*

MinimalYeast Medium	0. 67% Bacto-yeast nitrogen base w/o amino acids 2% Glucose supplemented with the amino acids when needed in the following concentrations: Leucine 30 mg/liter Uracil 20 mg/liter Tryptophan 20 mg/liter Histidine 20 mg/liter
YEPD	1% Bacto Yeast Extract 2% Bacto Peptone 2% Glucose For plates, 2% Bacto-agar was added
Sporulation Medium (SP_2)	0.3% Potassium Acetate 0.02% Raffinose
Presporulation Medium (PSP_2)	0.1% Yeast Extract 0.67% Yeast nitrogen base w/o amino acids 1% Potassium Acetate 0.5 M Potassium Phthalate buffer, pH 5.3

Five hundred microliters of the cell suspension served as 0 hour time point (vegetative cells) for β-galactosidase assays. Seven hundred and fifty microliters of the suspension was inoculated into 30 ml of sporulation (SP_2) medium, which was shaken at 30°C for 12 hours (Fig. 20). 0 hour and 12 hour cells were collected by centrifugation and resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2.7 ml/liter β-mercaptoethanol). Cells were collected by centrifugation in 1.5 ml microfuge tubes. One to two volumes of acid washed glass beads and 0.5 ml of Z buffer were added to the cell pellet. Cells were lysed by repeated cycles of vortexing for 30 seconds on the Big Vortexer (Glas-Col Apparatus Company, Terre Haute, IN) and incubation on ice for 30 seconds. For complete cell lysis vegetative cells required between 6-8 cycles, while sporulating cells required between 12-15 cycles. Cell lysis was monitored by viewing cells with a light microscope under 40X magnification. β-galactosidase reactions consisted of 5 to 300 μl of crude extract and Z buffer to bring the volume to 400 μl. Prior to the addition of substrate, reactions were incubated at 30°C for 10 minutes. The reaction was initiated by adding 0.1 ml of 4 mg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and incubated at 30°C. β-galactosidase converts ONPG, an analogue of its actual substrate lactose, into galactose and ortho-nitrophenol, a yellow compound which absorbs at 420 nm. The reaction was stopped when the absorbance at 420 nm was between 0.2-0.8 by adding 0.5 ml of 1 M Na_2CO_3. Absorbance was measured in the Beckman DU-8 spectrophotometer at 420 nm and at 550 nm to correct for cell debris. A spectrophotometric blank consisted of 0.4 ml of Z buffer, 0.1 ml of 4 mg/ml ONPG and 0.5 ml of 1 M Na_2CO_3.

Protein concentration of the extracts was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Crude extract (5μl) was mixed with in sterile water to final volume of 800 μl to which 200 μl of Bradford reagent was added. This mixture was incubated at room

Figure 20: Culture Conditions for Reporter Assays and Sporulation Measurements. All *S. cerevisiae* cultures used in this study were cultured under the same conditions unless otherwise stated. Cells were cultured in YEPD until the cell density was 2×10^7 cells/ml. A 1:25 dilution was made in PSP_2. After 12 hours the cells were washed twice in equal volumes of sterile H_2O. Forty percent of the cells were used for 0 hour assays and sixty percent shifted to SP_2 medium. Cells were washed once in equal volume of sterile H_2O prior to the 12 hour assay. Ten percent was allowed to sporulate in SP_2 medium to determine sporulation efficiency.

Transformant single colony

↓

24 hours in minimal glucose medium

↓

10 hours in YEPD + Trp medium

↓

10-12 hours in PSP_2 medium

↓

0 hour β-galactosidase assay and SP_2 shift to medium

↓

12 hour β-galactosidase assay

↓

Sporulation efficiency 24 hours after shift to SP_2 medium

temperature for 5 minutes. Absorbance was measured at 595 nm. The spectrophotometric blank consisted of 800 µl of sterile water and 200 µl Bradford reagent.

β-galactosidase specific activity was calculated by a two step procedure. First, the protein concentration was calculated based on the following equation:

$$\frac{A_{595} - 0.0855}{44.6} \times \text{dilution factor} = \text{mg/ml protein}$$

where A_{595} is the protein absorbance at 595 nanometers. The equation was derived from a linear regression analysis of a standard curve where 44.6 is the slope and 0.0855 is the X intercept.

The specific activity of β-galactosidase in Miller units/mg was determined according to the following formula:

$$\frac{1000\,[\,A_{420} - (2.5 \times A_{550})]}{\text{ml crude extract} \times \text{time(min)} \times \text{mg/ml}} = \text{Miller units/mg}$$

where A_{420} and A550 equal absorbance at 420 and 550 nm, respectively, minutes (min.) equal reaction time, ml equals amount of crude extract used, and mg/ml equals protein concentration. The A_{420} measurement reflects absorbance due to ortho-nitrophenol and cell debris; measurement at A_{550} detects cell debris only. The correction factor 2.5 in this equation was changed to 2.0 for calculating the specific activity for sporulation extracts. This substitution is necessary due to difference in the absorbance of cell debris in vegetative and sporulating cells.

A small portion of each SP_2 culture was incubated overnight at 30°C to allow the formation of mature asci. Sporulation efficiency was determined by light microscopic examination of 100 to 150 cells per culture at 100X magnification.To determine sporulation

efficiency the number of two-spored and four-spored asci were divided by the total number of cells counted and multiplied by 100.

4.2 Preparation of Competent *E. coli* NM522 cells

A 25 ml Luria Broth (Table 7) culture was started with a single NM522 [F'lacI^{q}Δ(*lacZ*)M15, proA, proB/supE, thi, Δ(lac-proAB), Δ(hsdMS-mcrB)5] colony and incubated overnight at 37°C with shaking. The culture was then diluted 1:40 in 500 ml of Luria Broth and incubated at 37°C with shaking until the absorbance at 550 nm was between 0.4 and 0.5. The culture was immediately chilled by swirling vigorously in an ice water bath for 10 minutes. Cells were harvested by centrifugation in a Sorvall centrifuge at 4000 × g for 10 minutes at 4°C. Cells were resuspended in 250 ml of ice cold 100 mM $MgCl_2$, 10 mM Tris, pH 8.0, and incubated on ice for 30 minutes with occasional swirling. Cells were collected by centrifugation at 4000 × g for 10 minutes for 4°C and resuspended in 250 ml of 100 mM $CaCl_2$, 10 mM Tris pH 8.0. After a 20 minute incubation on ice, cells were collected by centrifugation and resuspended in a final volume of 20 ml of ice cold 100 mM $CaCl_2$, 10 mM Tris pH 8.0, 20% glycerol. The cells were distributed in 1 ml aliquots into sterile tubes and incubated on ice for 1 hour. To maintain competency the cells were quick frozen in liquid nitrogen and stored at -70°C.

Table 7: Media for *E. coli*

Rich Medium + Glucose	1% Tryptone 0.5% Yeast Extract 0.5% Sodium Chloride 0.2% Glucose
Luria-Bertani (LB)	1% Bacto-Tryptone 0.5% Bacto Yeast Extract 1% Sodium Chloride For plates, 1.5% Bacto-agar was added.

Medium for growth of bacteria was supplemented with 50 μg/ml or 100μg/ml of ampicillin as recommended.

4.2a Bacterial Transformation

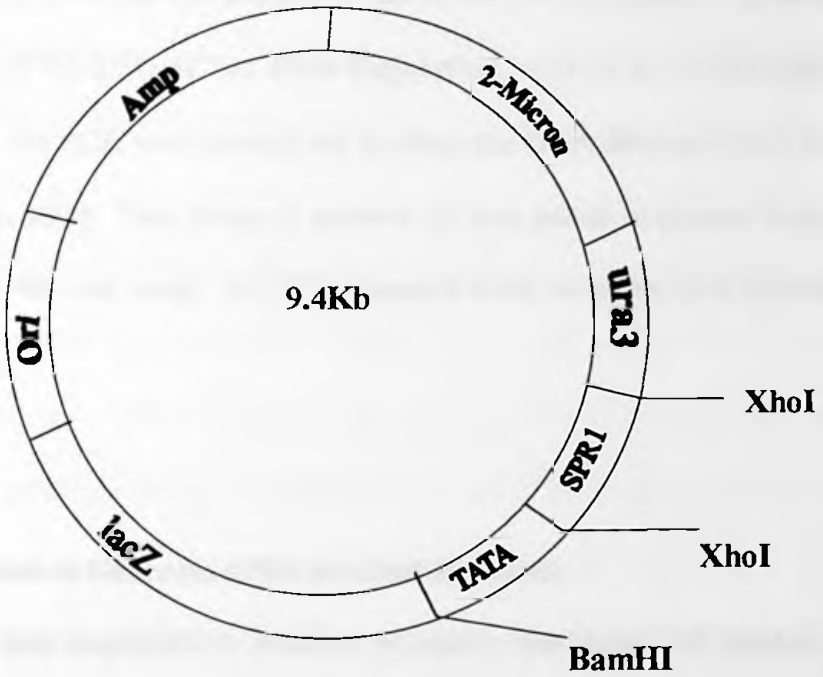
Competent *E. coli* NM522 cells were thawed on ice for 1 hour. Aliquots of 150-200 µl of cells were placed in 13 × 100 glass tubes. Ligation reactions (5 µl) were added to each tube and kept on ice for 45 minutes. After incubation at 42°C for 2 minutes, 1 ml Luria Broth was added and the cultures rotated on a wheel for 1 hour at 37°C. One hundred microliters of the transformation mixture was plated out on Luria Broth + 50 µg/ml ampicillin plates and incubated at 37°C for 10-12 hours before colonies were picked for plasmid DNA isolation.

4.2b Reporter Gene Vector

A 9.4 kb replicating vector, p312ΔX, was used to construct *lacZ* reporters; In Fig. 21, an *SPR1* insert is present for illustration purposes. This plasmid was made by converting the SmaI site of plasmid LG312 (Guarente and Mason, 1983) into an XhoI site and removing intervening UAS sequences by digestion with XhoI. The resulting plasmid p312ΔX has an UAS-less *CYC1* promoter flanked by a XhoI site at -3387 and a BamHI site at -3641 (Fig. 21). A *lacZ* reporter gene is fused to the *CYC1* promoter. Fragments from *SPR1* or *SPR2* promoters were inserted into either the XhoI site or between XhoI and BamHI sites, depending on the type of fusion being constructed. p312ΔX contains selectable markers for *E. coli* (ampicillin resistance) and *S. cerevisiae* (*URA3*). This plasmid also contains origins of replication for both organisms, oriC for *E. coli* and 2 micron origin of replication for *S. cerevisiae*.

Figure 21: Yeast Shuttle Vector 312ΔX. This plasmid was used in the construction of *SPR1-lacZ* translational fusions and *SPR1-* and *SPR2-CYC1-lacZ* transcriptional fusions.

312ΔX



4.3 Polymerase Chain Reaction (PCR)

The PCR was used to generate fragments of the *SPR1* and *SPR2* promoters for construction of reporter gene fusions. Primers were designed using the OLIGO v 4.0 software. Reaction cycles consisted of three steps: (1) denaturation at 94°C for 1 minute, (2) annealing between 50-55°C for 1 minute, depending on the primer pairs and (3) extension at 72°C for 2 minutes. Each cycle was repeated 30 times. A typical 100 µl reaction mixture contained 1 µg template DNA (e.g., plasmid pCG33), 100 pmoles of upper and lower primers, 2 µl each of 10 mM dATP, dCTP, dGTP, dTTP, 2.5U of Taq DNA Polymerase, 4-16 µl of 25 mM $MgCl_2$, and 10 µl of 10X PCR buffer. The PCR were carried out in either the DNA thermal cycler 480 or in the GeneAmp PCR System 9600. Two drops of mineral oil was added to prevent evaporation when DNA thermal cycler 480 was used. All PCR reagents were obtained from Perkin Elmer (Foster City, CA).

4.3a Asymmetric PCR Used to Generate *SPR1* Internal Deletions

Asymmetric PCR was employed to produce primarily one strand of product. PCR reactions were set up as described previously, but without the lower primer. Ten cycles were done in the absence of the lower primer to amplify single stranded template with *SPR1* sequences in the construction of reporters, SPR1/151-64 and SPR1/93-64. Thirty-five additional cycles were done after the lower primer was added to the reaction mix (McCabe, 1990). These reactions were performed under the following conditions on a Perkin Elmer GeneAmp PCR System 9600:denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, extension at 72°C for 1.5 minutes; linked to 72°C for 7 minutes for a final extension after cycling.

4.3b Construction of *SPR1-lacZ* Translational Fusions

A series of 5' nested translational fusions of *SPR1* were made on average every 61 bp using different upper primers and the SPR1/768BglII lower primer (Table 8). Upper and lower PCR primers were designed with XhoI and BglII sites to facilitate directional cloning into p312ΔX. PCR fragments were digested with XhoI and BglII according to the manufacturer's instructions (Life Technologies, Gaithesburg, MD). Plasmid p312ΔX was digested with XhoI and BamHI. All translational fusions contain an *SPR1* promoter fragment and its ATG fused in-frame to the *lacZ* reporter gene (Fig. 22a). After digestion, *SPR1* fragments and plasmid DNA were purified by extraction with phenol-chloroform and precipitated with 0.1 volume 3M sodium acetate, pH 5.4, and 2 volumes of 100% ethanol. The PCR fragments and plasmid DNAs were dissolved in deionized H_2O and mixed to give various ratios of vector to insert. Ligation reactions were initiated by addition of T4 DNA ligase in a final volume of 10-15 μl and incubated at 16°C for 4 hours. Low TE (5 μl) was added to the ligation reaction prior to transformation. Ligation products were transformed into 150-200 μl of competent NM522 bacterial cells. Ampicillin-resistant colonies were screened for insert-positive clones by restriction digestion and analysis on agarose gels. The presence of insert was confirmed by DNA sequencing.

4.3c Construction of Internal Deletions Reporters

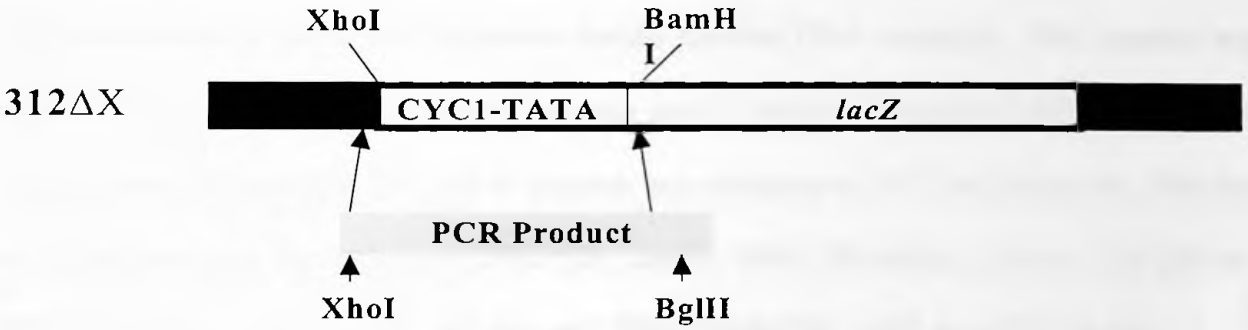
Four internal deletions in the region between -304 and -211 were constructed by recombinant PCR amplification (Higuchi, 1990). First, fragments on either side of the deletions were made via PCR using the *SPR1* gene (on plasmid pCG33) as the template. Next both products were gel purified and used as template in a second round of PCR amplification, which

Table 8: *SPR1* Primers Used in the Construction of *SPR1-lacZ* Translational Fusions. Primer names indicate the position of the first base pair at the 5' position, where +1 is the translation start. SPR1/BglII 768L containing a BglII site for subcloning purposes was used as the lower primer in all constructs. Both BglII and XhoI sites are underlined. U = Upper, L = Lower

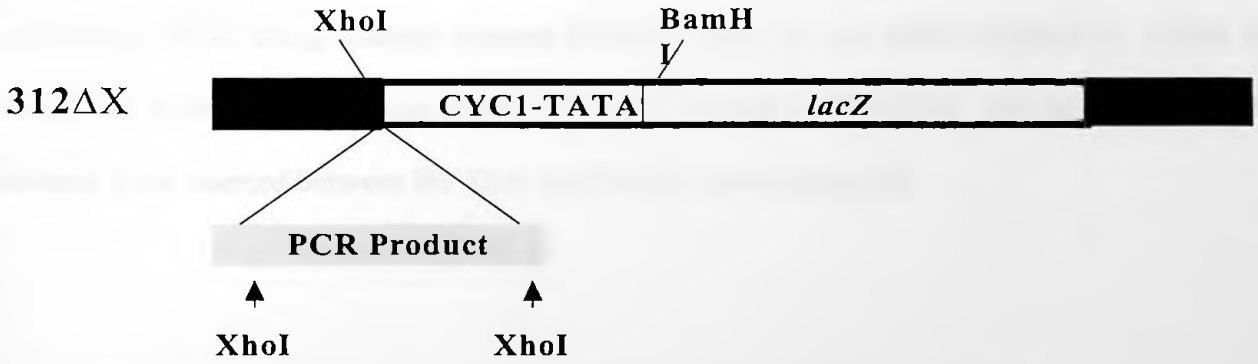
Name	Sequence
SPR1/762U	CGGCGATCCTCGAGTAAAACAACTTGAAAA
SPR1/731U	GAGGATGGACTCGAGTCATAAGGTA
SPR1/524U	AGAAGTAAGGCTCGAGACTGTAAAAG
SPR1/329U	GGTATCAAAAAGCCTCGAGGTTCATT
SPR1/268U	GGTCTCTGCCAGCTCGAGAAAAAGCG
SPR1/151U	CAGTTGCCACCCCTCGAGTAGCAACT
SPR1/93U	TTTACCCTTCTCGAGTAATTGTTCAAT
SPR1/BglII 768L	GCCCTCTGAACAGATCTCATTTACTTCT

Figure 22: Construction of Transcriptional and Translational Fusions. Part (**a**) illustrates a translational fusion, which contains portions of the 5' regulatory region, the promoter of the *SPR1* gene and its ATG fused to the *lacZ* reporter gene. The *CYC1* TATA is replaced by the insert. Part (**b**) illustrates a transcriptional fusion, made by inserting the 5' regulatory sequences upstream of the *CYC1* TATA element. In this type of fusion the *CYC1* TATA is retained.

(a) Schematic of *SPR1-lacZ* translational fusions



(b) Schematic of *SPR1-CYC1-lacZ* transcriptional fusions



used only the external primers (see Fig. 23 for schematic). A deletion is generated because the inside primers (which are positioned on the boundaries of the deletion) contain complementary base pairs which facilitates annealing of the primary products to each other. For the second stage PCR, a solution containing all the components except the external primers was cycled 5 times at 94°C for 45 seconds; annealing was performed at 40°C for 45 seconds to anneal the upper strand from the 5' PCR fragment and the lower strand from the 3' fragment. A 72°C extension step added nucleotides to the 3' end to make a double stranded DNA molecule. This reaction was followed by the addition of the external primers and 15 additional cycles of 94°C denaturation for 45 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 90 seconds. The first three deletions were derivatives of SPR1/524, namely SPR1/293del236, -294 to -234 (60 bp); SPR1/303del266, -304 to -265 (40 bp) and SPR1/294del276, -295 to -275 (20 bp). These deletions were made using the external primers SPR1/524U and SPR1/768L (Table 8), along with a unique set of inside primers designed to generate the desired deletion (Table 9, rows 1-6). A fourth deletion SPR1/251del211, spanning -251 to -211, was also constructed employing recombinant PCR, using internal primers SPR1/251del211U and SPR1/252del211L (Table 9, rows 7 and 8) and upper external primer SPR1/329 instead of SPR1/524. All recombinant PCR products were inserted between the XhoI and BamHI sites of p312ΔX.

4.3d Construction of SPR1/151-64 and SPR1/93-64 Translational fusions

Inserts SPR1/151-64 and SPR1/93-64 were generated by recombinant PCR using the internal primers listed in Table 9, rows 9-12. SPR1/64XhoI (Table 9, row 13) and SPR1/768BglII (Table 8) were used along with different sets of internal primers to make the

Figure 23: Recombinant PCR Used to Generate Internal Deletions. (A) PCR of the sequences flanking the potential deletion, using two different sets of primers. (B) Gel purified products are mixed, denatured and renatured. Extension occurs at the 3' end of the renatured products, in the absence of the outside primers. (C) The extended products containing the deletion of interest are amplified by PCR using the outside primers.

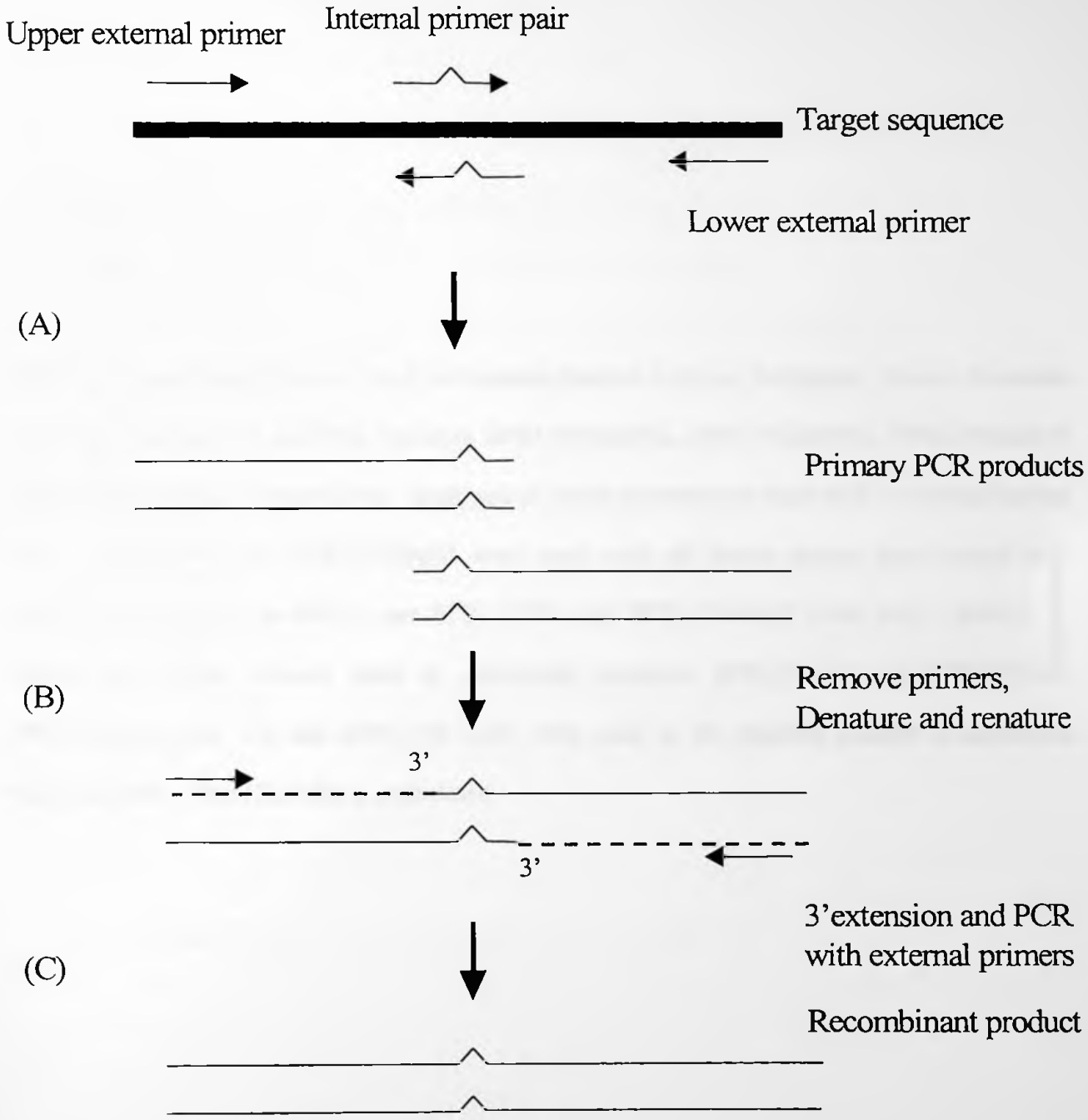

Upper external primer
Internal primer pair
Target sequence
Lower external primer
(A)
Primary PCR products
(B)
Remove primers,
Denature and renature
3'
3'
3'extension and PCR
with external primers
(C)
Recombinant product

Table 9: Sequences of Primers Used to Generate Internal Deletion Reporters. Rows 1-8 contain inside primers used to generate reporters SPR1/293del236, SPR1/303del266, SPR1/294del276 and SPR1/251del211, respectively. Each pair of inside primers was used with an external primer pair. SPR1/524U and SPR1/768BglII were used with all inside primer pairs except with SPR1/251del211U/L, in which case SPR1/329U and SPR1/768BglII were used. Rows 9-12 contain the inside primers used in generating reporters SPR1/93-64 and SPR1/151-64. SPR1/64XhoI (row 13) and SPR1/768 BglII were used as the external primers in generating these reporters. The XhoI site is underlined.

Name	Sequence
SPR1/293del236U	ACTACAGCGACTCTGAAGCGGCG
SPR1/293del236L	CGCCGCTTCAGAGTCGCTTGTAGT
SPR1/303del266U	TCATTATTTGTCCCACTAGAAAAAGCGGTTACC
SPR1/303del266L	GGTAACCGCTTTTTCTAGTGGGACAAATAATGA
SPR1/294del276U	CACTACAAGCGACTTGCCAGCTTAAGAAAA
SPR1/294del276L	TTTTCTTAAGCTGGCAAGTCGCTTGTAGTG
SPR1/251del211U	AAGAAAAAGCGGTTAAATTTGTCAGTAATG
SPR1/251del211L	CATTACTGACAAATTTAACCGCTTTTTCTT
SPR1/93 Upper	CGACACTAAAAGTCTTTTAATTGT
SPR1/93 Lower	ACAATTAAAAGACTTTTAGTGTCG
SPR1/151 Upper	CGACACTAAAAGTTTTGTAGCAAC
SPR1/151 Lower	GTTGCTACAAAACTTTTAGTGTCG
SPR1/64XhoI Upper	CACTACAAGCGACTCGAGTATTTT

desired deletions. Recombinant PCR products were digested with BglII and XhoI prior to subcloning between the BamHI and XhoI sites of p312ΔX. Standard ligation procedures are described in section 4.4b.

4.3e Construction of MSE-*SPR1-lacZ* Fusions

MSE-*SPR1-lacZ* reporters were made by inserting chimeric PCR products containing the *SPR2* MSE and portions of the *SPR1* promoter upstream and in-frame with the *lacZ* gene in p312ΔX. Each upper primer had the following components reading from the 5' end: a 7 bp spacer to enhance restriction enzyme digestion, an XhoI site for subcloning purposes, 26 bp of *SPR2* which include an MSE and 21 bp of *SPR1* sequences depending on the primer binding site; i.e. -151 or -93 of *SPR1*. The sequence of each primer is as follows: SPR1/93-MSE, CGCTAGC CTCGAGTTTTTGTGTCCCCATGGTCTTTTAATTGTTCAATTGCG and SPR1/151-MSE, CACGATCCTCGAGTTTTTGTGCCCATGGTTTTGTAGCAACTTCCATGCG. The lower primer SPR1/768BglII (Table 8) had a BglII site for subcloning purposes. The PCR were performed under the following conditions: denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 1.5 minutes. This was linked to 72°C for 7 minutes for a final extension after cycling on a Perkin Elmer GeneAmp PCR System 9600. The chimeric PCR products were inserted between the BamHI and XhoI sites of p312ΔX.

4.4 Transcriptional Fusions

SPR1 and *SPR2* transcriptional fusions were made by inserting either duplex synthetic oligonucleotides or PCR products (promoter fragments) into the XhoI site of the enhancerless *CYC1-lacZ* reporter in p312ΔX. Transcriptional fusions contained the *CYC1* TATA element and the *SPR1* or *SPR2* fragment of interest inserted at the XhoI site (Fig. 22b). In instances when PCR was used to generate the DNA fragment from plasmid clones, primer pairs were employed as described below in section 4.4c. The PCR products were digested with restriction enzymes and subcloned into p312ΔX. Restriction digests were performed on positive clones to determine orientation.

4.4a Generation and Purification of Duplex Oligonucleotides for Ligation

Equimolar amounts (4 nmoles) of the oligonucleotides were added to a solution with a final concentration of 50 mM NaCl. These oligonucleotides contained either *SPR1* or *SPR2* promoter sequences and XhoI overhangs for subcloning purposes. Low TE (10 mM Tris, 0.1 mM EDTA) was used as the buffer to make up the final volume to 50 µl. Oligonucleotides were annealed on a PTC-200 thermal cycler programmed for 95°C for 5 minutes, 65°C for 5 minutes, and slow cooling from 65°C to 4°C at one degree per minute.

The annealed fragments were purified by electrophoresis at 100 V on a 2% Nu Sieve gel and electroeluted as described below. The gel was stained with 30 µM ethidium bromide for 5 minutes. DNA duplexes were cut from the gel after visualizing under UV light and the gel pieces were soaked in 1X low salt buffer (20 mM Tris, pH 8.0; 0.2 mM EDTA; 5mM NaCl). Duplex DNA was then electroeluted using an IBI model 4600 DNA electroelutor. The eluted

duplex was precipitated by adding one tenth volume 3M sodium acetate, pH 5.4, and either two volumes of 100% ethanol or one volume of isopropanol, and incubating at -20°C overnight. The DNA was collected by centrifugation; pellets were washed with 75% ethanol and dissolved in 18 µl of sterile H_2O, then made up to 20 µl by adding 1 µl low TE and 1 µl of 0.2 M NaCl. The duplexes were reannealed in the PTC-200 thermal cycler by incubating at 65°C for 5 minutes followed by cooling from 65°C to 4°C at one degree per minute.

4.4b Ligation

Ligation of duplex oligonucleotides to plasmid 312ΔX was performed in a final volume of 10-15 µl by a linker tailing protocol. The reaction volume was kept as low as possible to enhance the interaction between the insert and vector molecules. A standard ligation reaction contained 2.25 µmoles of duplex oligonucleotide, 1X T4 DNA ligase buffer, 1µl T4 DNA ligase (1 unit/µl; Life Technologies, Gaithesburg, MD) and 20 µg of plasmid DNA digested with XhoI. Ligation was performed on the Perkin Elmer GeneAmp PCR System 9600 thermal cycler which was programmed for incubation at 16°C for 1 hour, melting at 65°C for 5 min, and cooling from 65°C to 16°C, at a degree per minute. When the reaction reached 16°C, 2 µl of 5X T4 ligase buffer and 1 µl of T4 ligase were added and incubation was continued at 16°C for 1 hour. This additional step of melting and cooling increased ligation efficiency, presumably by removing a second insert that might inhibit the ligation reaction.

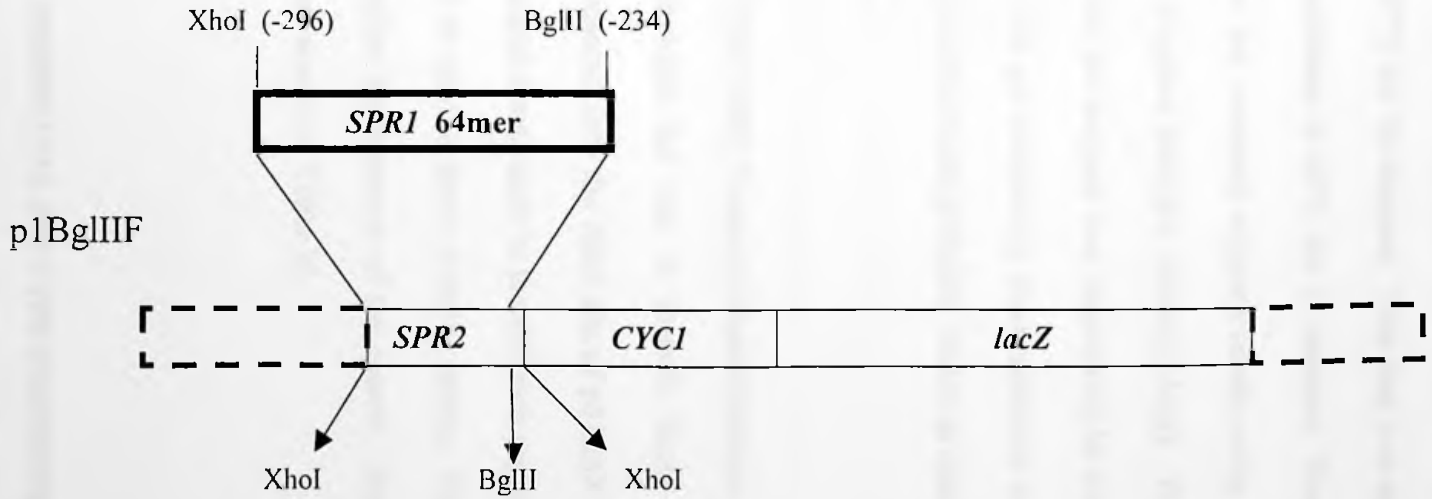
4.4c Construction of SPR1/268-141 and SPR1/268-192 Transcriptional Fusions

The PCR was used to generate *SPR1* promoter fragments for constructing the *SPR1-CYC1-lacZ* fusions. XhoI and SalI sites were designed into the upper and lower primers respectively. Primer sequences were as follows: SPR1/268U, GGTCTCTGCCAGCTCGAGAA AAAGCG, SPR1/2141L, GGACACAAAGGTCGACAGCATTAC, SPR1/192L, TGAGGTTG GTCGACCATGGAAGT. The reaction was performed under the following conditions: denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension for 72°C for 1.5 minutes. The PCR products were digested with XhoI and SalI and subcloned into the XhoI site of p312ΔX.

4.4d Construction of the SPR1/296-234F Reporter

Attempts to ligate the annealed, gel purified 64 bp duplex oligonucleotide into the XhoI-digested p312ΔX vector failed to yield any positive clones on several attempts. Therefore, this clone was obtained by a forced cloning strategy. SPR1/296-234F contains a 64 bp sequence of the *SPR1,* gene spanning from -296 to -234, inserted into the XhoI and BglII sites of pIBglIIF. The upper oligonucleotide *SPR1* 296-234 U (*SPR1*-64mer), TCGAGCTTATTTTTGGCGGT CTCTGCCAGCTTAAGAAAAAGCGGTTACCAAACGACACTAAAA, and the lower oligonucleotide *SPR1* 296-234 L (*SPR1*-64mer), GATCTTTTAGTGTCGTTTGGTAACCGCTT TTTCTTAAGCTGGCAGAGACCGCCAAAAATAAGC, were annealed as described in section 4.4a. An *SPR2-CYC1- lacZ* reporter vector (p1BglIIF) with XhoI and BglII sites in the promoter was used as the backbone for this ligation. Plasmid 1BglIIF (Fig. 24) was digested sequentially first with BglII and then with XhoI. Sequential digestion was necessary to limit digestion at a

Figure 24: Construction of the *SPR1* Transcriptional Fusion (SPR1/296-234). The *SPR2-CYC1-lacZ* fusion p1BglIIF was used as the backbone. BglII and XhoI sites were used to subclone the *SPR1* insert by replacing the *SPR2* insert. The hatched regions represent flanking sequences of p312ΔX.


XhoI (-296)
BglII (-234)
SPR1 64mer
p1BglIIF
SPR2
CYC1
lacZ
XhoI
BglII
XhoI

second XhoI site adjacent to the BglII site. The linearized vector was dephosphorylated by treating with1 μl of calf alkaline phosphatase (1 unit/μl, Boehringer Mannheim, Indianapolis, IN) and incubating at 37°C for 10 minutes. This step was repeated a second time prior to heat inactivation of the phosphatase at 65°C for 15 minutes. Phosphate groups were enzymatically added to the 5' ends of the annealed oligonucleotide using 1 μl of T4 polynucleotide kinase (10,000 units/ml, New England BioLabs, Beverly, MA). The kinase reaction was incubated at 37°C for 30 minutes, and the enzyme was inactivated by heating at 65°C for 20 minutes. The phosphorylated duplex was gel purified by electrophoresis on a 2% Nu Sieve gel, electroeluted, and ligated into the dephosphorylated p1BglIIF vector as described in section 4.4a.

4.4e Construction of *SPR2* MSE Transcriptional Fusions

In order to determine the role of specific bases in the *SPR2* MSE, mutant MSE oligonucleotides were inserted into the XhoI site of p312ΔX. Since these inserts were less than 20 bp in length, bp changes were made in the GC rich region to generate restriction sites (DraI and BglII) which could be used to detect positive clones. Digestions by the respective enzymes were performed to confirm the presence of the inserts. Sequences of the SPR2 MSE mutant oligonucleotides used are shown in Table 10.

4.5 Sequencing

4.5a Purification of Template DNA and Cycle Sequencing

The Wizard miniprep kit (Promega, Madison, WI) was used according to manufacturer's

Table 10: Oligonucleotide Sequences Used in the Construction of Transcriptional Fusions. Upper and lower primers were annealed before ligation to make *SPR2*(MSE)-*CYC1*-lacZ constructs. The core *SPR2* MSE is underlined and the DraI and BglII restriction sites are shown in bold.

Name	Sequence
SPR2/R1 Upper (wild type)	5' TCGATTTTTGTGCTACGa 3'
SPR2/R1 Lower (wild type)	5' TCGatCGTAGCACAAAAA 3'
SPR2/R2draI Upper (mutant)	5' TCGAGTTTTTGTG**TttaAa**GG 3'
SPR2/R2draI Lower (mutant)	5' TcgaCC**tTtaaA**CACAAAAAC 3'
SPR2/R2bglII Upper (mutant)	5' TCGAGTTTTTGTG**TaagATct** 3'
SPR2/R2bglII Lower (mutant)	5' TCG**agATctt**ACACAAAAAC 3'

instructions to purify plasmid DNA from 3 ml of bacterial cells. Each sequencing reaction contained 40 pmoles of primer, 10 μg of template DNA and 4 μl of ABI dye terminator ready mix with the AmpliTaq polymerase (Perkin Elmer). The thermal cycler was programmed for 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes; each cycle was repeated 25 times. Excess dideoxy terminators were removed by passing sequencing products through Centri-Sep spin columns (Princeton Separations, Adelphia, NJ). The columns were reconstituted with 0.8 ml of reagent grade H_2O and hydrated by inverting and vortexing briefly. The columns were incubated for a minimum of 30 minutes at room temperature. After bubbles were removed, the cap and the stopper were removed sequentially. Excess fluid was drained first by gravity and then by centrifugation at 735 × g for 2 minutes. The dye terminator reaction mix was placed carefully onto the center of the gel bed and spun at 735 × g for 2 minutes to collect the sample. The sample was precipitated with three volumes of ethanol and stored at -20°C until further use. The pellet was dissolved in 25 μl of template suspension reagent (Perkin Elmer, Foster City, CA) and incubated at room temperature for 10 minutes. Samples were heated to 95°C for 2 minutes and applied to an ABI 310 automated sequencer (Perkin Elmer, Foster City, CA).

4.6 Mobility Shift Assays.

4.6a Labeling Probes with ^{32}P

Mobility shift assays were performed to determine if specific promoter sequences were bound by sporulation-specific DNA binding proteins. Oligonucleotides were synthesized at the Marshall University Core Facility on an Applied Biosystems 394 DNA/RNA synthesizer.

Equimolar amounts of upper and lower oligonucleotides (Table 11) were annealed on the Perkin Elmer GeneAmp PCR System 9600 as previously described in section 4.4a. Three nmoles of duplex oligonucleotides were labeled with ^{32}P-ATP in reactions containing 3 µl 10X kinase buffer, 2 µl T4 polynucleotide kinase (New England BioLabs, Beverly, MA) and 2 µl of ^{32}P-ATP (10 µCi/µl) in a final volume of 30 µl. The reaction was incubated at 37°C for 30 minutes and the kinase was inactivated by incubating at 70°C for 10 minutes. G-25 Centricon columns (Boehringer Mannheim, Indianapolis, IN) were used to remove unincorporated nucleotides. ^{32}P-labeled DNA duplexes were purified by electrophoresis on a 6% denaturing polyacrylamide gel. The appropriate fragments were cut out of the gel, placed in a 5-cc syringe barrel and sheered by centrifugation at 1650 × g through an 18 gauge needle for 10 minutes. Elution buffer (10 mM Tris, pH 7.5; 0.1 mM EDTA and 0.3 M LiCl) was added to the DNA/gel mix and shaken at 37°C for either 3 hours or overnight. The gel/buffer mixture was applied to a Quik-Sep funnel by centrifugation at 1650 × g for 10 minutes. Eluted DNA was precipitated by adding 0.1 volume of 3M sodium acetate, pH 5.4, and 2 volumes of 100% ethanol and incubating for 1 hour or overnight at -20°C; the pellets were resuspended in 20 µl of low TE. In some instances the labeled probe was passed through two G-25 columns, instead of gel purification, in order to reduce the loss of labeled probe. Before use in gel shift reactions, ^{32}P-labeled probes were diluted 1:4 in buffer A'(20 mM Hepes, pH 7.9, 150 mM KCl, 20% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol) which contained the following protease inhibitors: 5 µg/ml aprotinin, 17 µg/ml PMSF; 1 µg/ml leupeptin.

Table 11: DNA Probes Used in EMSAs. All probes except SPR1/GSS 259 upper and lower are *SPR1* probes. Mut C upper and lower are mutant MSE sequences containing point mutations; the point mutations are underlined. SPR1/GSS 259 upper and lower are *SPR2* MSEs. The length of each probe is indicated beside the given name of the oligonucleotide. Putative *SPR1* binding elements are in bold.

Name	Sequence
SPR1/GSS 294 U 60bp	**TCTTATTTTTGGCGGT**CTCTGCCAGCTTAAGAAAAAGC GGTTA**CCAAACGACACTAAAA**G
SPR1/GSS 294 L	CTTTTAGTGTCGTTTGGTAACCGCTTTTTCTTAAGCTGGC AGAGACCGCCAAAAATAAGA
SPR1/GSS 251 U 41bp	**CCAAACGACACTAAAA**GAAGCGGCGCGTCTGGTTCAT TAGA
SPR1/GSS 251 L	TCTAATGAACCAGACGCGCCGCTTCTTTTAGTGTCTTTT GG
SPR1/GSS 293 U 42bp	**CTTATTTTTGGCGGT**CTCTGCCAGCTTAAGAAAAAGCG GTTA
SPR1/GSS 293 L	TAACCGCTTTTTCTTAAGCTGGCAGAGACCGCCAAAAA TAAG
SPR1/GSS 259 U 31bp	TTTTTGTGGCTACGTTTTTGTGTCCCATGGC
SPR1/GSS 259 L	GCCATGGGACACAAAAACGTAGCCACAAAAA
Mut C U 11bp	GCCAGTAACAC
Mut C L	GTGTTACTGGC

4.6b Preparation of Crude Protein Extract for EMSAs

S. cerevisiae strains GKY5 and YSC508 (Table 5) were grown as previously described in section 4.1a. Cells were disrupted by adding one to two volumes of glass beads and 0.5 ml of glass bead breaking buffer A'. Protease inhibitors were added just prior to use to the buffer to a final concentration of 17 μg/ml of PMSF, 1 μg/ml of leupeptin and 5 μg/μl aprotinin. Cells were lysed by vortexing twice for 10 minutes at 4°C. Cell lysis was monitored by viewing cells with a light microscope under 40X magnification. The extracts were either used immediately or were stored at -70°C.

4.6c Preparation of Recombinant Ndt80 Protein for EMSAs

Recombinant Mbp and Mbp-Ndt80 fusion protein were purified from two protease-deficient bacterial strains NB42/p163 and NB42/p164, containing expression plasmids pMAL-p2 and MBP-*NDT80*, respectively. These strains were obtained from Ira Herskowitz at University of California, San Francisco. Strains were first cultured in 20 ml of Luria Broth + ampicillin (100 μg/ml) (Table 7) by shaking at 37°C overnight. One liter of Luria Broth + ampicillin was inoculated with the 20 ml starter culture and incubated at 37°C with shaking for 2 hours. Expression of Mbp or Mbp-Ndt80p was then induced by addition of IPTG (isopropylthio-β-D-galactoside) to a final concentration of 0.3 mM, incubating at 37°C with shaking for 3 hours. Cells were harvested by centrifugation at 3300 × g for 20 minutes for 4°C in a Sorvall RC-5B centrifuge (Du Pont Instruments, Wilmington, DE). Cells were resuspended in 35 ml of PBS buffer (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2PO_4 $7H_2O$; 1.4 mM KH_2PO_4). Cells were

sonicated on an ultrasonic homogenizer (4710 series, Cole Parmer Instrument Co., Chicago, IL) five times for 20 seconds each. 10% Triton X-100 was added to the homogenate to a final concentration of 1%. The homogenate was mixed by rotation for 30 minutes, then subjected to centrifugation at 12,000 × g for 20 minutes. The supernatant was bound to 1 ml amylose resin suspension and placed on a rotor for an additional 30 minutes. The resin/ supernatant mix was diluted 1:1 with PBS and poured into a 3mm column. The bound resin was washed twice with 5 ml of PBS buffer and the Mbp fusion protein was eluted with 5 ml of 10 mM maltose in PBS. This step was repeated on the eluate to ensure the removal of additional unbound materials. Bradford assays were performed on wash and eluate fractions. Eluate fractions with the highest protein concentration were pooled and concentrated on centricon 30 columns (Perkin Elmer, Amicon Division). The purified protein was quantitated based on a BSA standard curve. Proteins were also analyzed on SDS-PAGE gels as described in section 4.9b.

4.6d Electrophoretic Mobility Shift Assays (EMSAs)

Binding reactions contained the ^{32}P-labeled probes and either crude vegetative or sporulation protein extracts or recombinant Ndt80p (Chu and Herskowitz, 1998). Each 25 µl reaction contained 1 µl of labeled probe (30,000-50,000 cpm), 20-25 µg of crude protein extracts or 2.8 µg of recombinant protein, 10 µl of buffer A', 1 µl of 1 µg/µl of dI/dC, 11 mM $MgCl_2$, 50 mM $ZnSO_4$, and 0.65 mM DTT. All reagents were mixed and incubated at 30°C for 50 minutes. In competition reactions, the unlabeled DNA competitor was incubated with the protein for 15 minutes before the labeled probe was added. The reactions were loaded onto an 8% native polyacrylamide gel and electrophoresed for four to five hours at 130 V. The gel was transferred

onto Whatman paper (3MM) and dried on a Savant SGD2000 slab gel dryer for 1 hour at 80°C. The gel was placed on a cassette and exposed to Kodak X-OMAT film for between 1 to 6 hours at room temperature; Kodak film was processed with a automatic film developer.

4.7 Western Blots and Protein Gels

4.7a Preparation of Denaturing Gel

Denaturing polyacrylamide gels were used to separate proteins based on size and determine the extent of purity. Western blots were then employed to identify the protein of interest using antibodies directed to a certain portion of the protein. The purity of the protein preparation was estimated by calculating the ratio of protein bound to Ndt80 antibody to non-specific protein present in the partially purified eluate samples. The final concentrations in the separating gel was 12.5% polyacrylamide, 10% bisacrylamide, 0.4M Tris (pH 8.7), 0.1% SDS and deionized H_2O. Polymerization was initiated by the addition of ammonium persulfate and TEMED. The gel was allowed to polymerize at room temperature for 1 hour. The final concentration of the stacking gel was 8% polyacrylamide, 0.2% bisacrylamide, 0.14 M Tris pH 6.9, 0.1 %SDS and sterile H_2O. Protein samples were heated at 100°C for 5 minutes prior to loading. The running buffer (200 mM glycine; 25 mM Tris; 3.5 mM SDS) was added to the upper and lower chambers. Two gels were simultaneously loaded and electrophoresed overnight at 46 V. One gel was processed for western blotting while the other was stained with Coomassie Brilliant Blue dye.

4.7b Procedure for Coomassie Staining a Protein Gel

The separating gel was placed in a tray containing Coomassie stain (0.025% Coomassie Brilliant Blue R250, 40% methanol, 7% acetic acid) and placed on a rocking platform at room temperature for 1 hour. The staining solution was removed, the gel was washed with 50 ml of high destain solution (40% methanol, 7% acetic acid), and soaked in high destain solution for 1 hour on the rocker, then the high destain solution was replaced with low destain solution (7% acetic acid, 5% methanol). Destaining with the low destain solution was repeated 3-4 times until the bands were visible. An image of the stained gel was obtained by scanning with an Epson ES 1200c scanner.

4.7c Western Blots: Transblotting and Immunoblotting

The polyacrylamide gel, six pieces of 3MM Whatman chromatography filter paper, and a piece of nitrocellulose were soaked in transfer buffer (20% methanol, 0.5% Tris, 3% glycine) for 10 minutes. Three pieces of Whatman paper soaked in transfer buffer were placed on a piece of soaked sponge inside the transblot apparatus, then the gel, nitrocellulose membrane, and the other three pieces of Whatman paper were added and covered with the second piece of sponge. The transblot was inserted in the chamber and proteins were transferred from the gel to the membrane by electrophoresis at 300 mAmps for 3 hours. The blot was incubated in 3% non-fat dry milk in TBST buffer (10 mM Tris, pH 8.0, 0.15 mM NaCl, 0.05% Tween) for 1 hour at room temperature. Next, the blot was incubated in 1:10,000 dilution of primary antibody (anti-MBP serum, New England BioLabs) in TBST containing 3% non-fat dry milk for 1 hour at room temperature. The blot was washed once in TBST for 10 minutes and incubated in 1:3000

dilution of peroxidase conjugated anti-rabbit IgG secondary antibody in 3% TBST (Sigma). The blot was washed with TBST three times for 10 minutes each. 4 ml of ECL solutions A and B (Amersham, Chicago, IL) were mixed and the blot was incubated for 1 minute. Fuji X-ray film was exposed for 1 minute or less and developed on the RGII developer (Fuji Photo Film USA, Inc.).

5.0 LITERATURE CITED

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