Avoiding unscheduled transcription in shared promoters:
Saccharomyces cerevisiae Sum1p represses the divergent gene pair SPS18-SPS19 through a midsporulation element (MSE)

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Abstract

The sporulation-specific gene SPS18 shares a common promoter region with the oleic acid-inducible gene SPS19. Both genes are transcribed in sporulating diploid cells, albeit unevenly in favour of SPS18, whereas in haploid cells grown on fatty acids only SPS19 is highly activated. Here, SPS19 oleate-response element (ORE) conferred activation on a basal CYC1-lacZ reporter gene equally in both orientations, but promoter analysis using SPS18-lacZ reporter constructs with deletions identified a repressing fragment containing a midsporulation element (MSE) that could be involved in imposing directionality towards SPS19 in oleic acid-induced cells. In sporulating diploids, MSES recruit the Ndt80p transcription factor for activation, whereas under vegetative conditions, certain MSEs are targeted by the Sum1p repressor in association with Hst1p and Rfm1p. Quantitative real-time PCR demonstrated that in haploid sum1D, hst1D, or rfm1D cells, oleic acid-dependent expression of SPS18 was higher compared with the situation in wild-type cells, but in the sum1Δ mutant, this effect was diminished in the absence of Oaf1p or Pip2p. We conclude that SPS18 MSE is a functional element repressing the expression of both SPS18 and SPS19, and is a component of a stricture mechanism shielding SPS18 from the dramatic increase in ORE-dependent transcription of SPS19 in oleic acid-grown cells.

Introduction

Divergent genes occur as two ORFs, one on each DNA strand, that are transcribed outwardly from a common promoter region delineated by the pair’s ATG start sites. The compact genome of the yeast Saccharomyces cerevisiae contains numerous divergent genes, which, in certain cases, are involved in the same cellular process. Simultaneous regulation of metabolically linked divergent genes is mediated by promoter elements that direct the transcriptional machinery towards each of the coding sequences. For example, GAL1 and GAL10 required for galactose breakdown are coordinated by UASG located between the genes (Johnston & Davis, 1984; West et al., 1984; Yocum et al., 1984). Other divergent systems, such as the one represented by the two sporulation-specific genes DIT1 and DIT2, use repressors for coordinated control. In this case, a DIT repressor element constituting the major negative regulatory site during vegetative growth (Bogengruber et al., 1998) exerts repression in conjunction with a midsporulation element (MSE) situated within an negative regulatory element (NRE_DIT) (Friesen et al., 1997). However, at least from...
the probable functions assigned to them in the yeast databases (YPD™, http://www.proteome.com; SGD™, http://genomewww.stanford.edu), it seems that in the vast majority of cases divergent genes encode proteins that are not involved in the same process.

Divergent genes could ostensibly be regulated from their common promoter region through elements operating unidirectionally to enhance transcription of only one gene at a time. In general, however, known regulatory transcription factors targeting promoter elements act in both orientations (Angermayr & Bandlow, 1997). This feature also holds true for the two-tracked activating mechanism in the respective promoters of the POT1/FOX3 and CTA1 genes that are induced on oleic acid medium (Einerhand et al., 1993; Filipits et al., 1993). Hence, to ensure temporal specificity, divergent gene systems that are guided by different transcriptional schedules must use dedicated stricture mechanisms.

The present study is concerned with the manner in which divergent gene promoters containing bidirectional elements mediate selective regulation in only one orientation. The SPS18–SPS19 gene pair was chosen as a test system because previous work demonstrated that despite being separated by a short 300-nucleotide promoter region, their individual transcription schedules vary significantly. For example, in diploids undergoing sporulation, SPS18 is highly transcribed between 5 and 11 h into the process, whereas SPS19 transcription is very much lower (Coe et al., 1993; Filipits et al., 1993). Hence, to ensure temporal specificity, divergent gene systems that are guided by different transcriptional schedules must use dedicated stricture mechanisms.

The SPS18–SPS19 promoter region also contains a functional MSE (Fig. 1) that responds to the transcription factor Ndt80p (Ozsolac et al., 1997; Chu et al., 1998). Certain MSEs additionally bind Sum1p – in combination with Hst1p (Xie et al., 1999; Pierce et al., 2003) and Rfm1p (McCord et al., 2003) – to repress genes under vegetative conditions. Indeed, both SPS18 and SPS19 are upregulated in the absence of Hst1p, but their unscheduled expression profiles do not resemble each other (Wyrick et al., 1999). To elucidate the mechanism repressing SPS18 when expression of SPS19 is induced, a set of deletions in the promoter region was constructed and their effect on SPS18 expression was determined. The action of Sum1p, Hst1p and Rfm1p on the SPS18–SPS19 intergenic region under oleic acid-induction conditions was also assessed. The results are discussed in terms of the shielding of genes in divergent systems from unscheduled transcriptional activation.

Materials and methods

Strains, plasmids, and oligonucleotides

The S. cerevisiae strains and plasmids used are listed in Tables 1 and 2, respectively. The Escherichia coli strain DH10B was used for all plasmid amplifications and isolations. Construction of the BJ1991-derived strains (Jones, 1977), BJ1991pip2Δ, BJ1991oaf1Δ, and BJ1991pip2Δoaf1Δ (Rottensteiner et al., 1996, 1997) or yAG259 and yAG561 (Gurvitz et al., 1997b), has been described. To generate strains yAG547, yAG554, yAG565, yAG557, and yAG569, the respective plasmids pAG528, pAG530, pAG536, pAG532, or pAG538 were linearized using StuI and verifiably integrated as a single copy (Southern, 1975) into the ura3 locus of BJ1991 wild-type (WT) cells (Chen et al., 1992). Strains yAG1310 and yAG1312 were constructed by integrating a single copy of StuI-linearized plasmids pAG534 or pAG536, respectively, into the ura3 locus of BJ1991pip2Δoaf1Δ. Strain yHPR1550 was constructed by introducing a single copy of a StuI-linearized pPS19 ORE:CYC1-lacZ plasmid into the ura3 locus of BJ1991 WT cells. The WT strain

Fig. 1. Scheme of the SPS18–SPS19 intergenic region incorporated into the reporter genes used. A 1.4-kb XbaI–SphI fragment including the shared SPS18–SPS19 promoter region and a portion of the reading frames of both genes was used as template for site-directed mutagenesis. The distance between the two ATG translational start codons is 300 bp. The terminal 3' G in the depicted sequence occurs 79 nucleotides upstream of the SPS18 ATG site, whereas the terminal 5' C is 130 bp upstream of the SPS19 ATG triplet. The sequences representing UAS1PS19 ORE, UAS1PS19, and SPS18 MSE, are indicated as boxes below the sequence. Boxed regions above the sequence represent mutations introduced into the promoter that was incorporated within the various reporter genes used. An XhoI restriction site (CTCGGAG) was substituted for the boxed DNA sequences designated M1 and M3, whereas regions designated M2, M4, and M5 were deleted. TATA-box sequences TATAAA or TATAAG occur 61 and 103 nucleotides 5' of the SPS18 and SPS19 ATG start codons, respectively.
Strains yAG1193 and yAG1230 were constructed by site-directed mutagenesis. The mutated DNA was verified by nucleotide sequencing. Construction of plasmid pAG534 containing the WT SPS18 promoter fused with the lacZ gene in YIp356R (Myers et al., 1986) was outlined previously (Gurvitz et al., 1997b). Plasmids pAG528, pAG530, and pAG532 consisted of the respective M1, M2, and M4 mutated promoters. Plasmids pAG536 and pAG538 (M3 and M5 mutated promoters, respectively) were constructed here. Plasmid pPS19 ORE: CYC1-lacZ was constructed from pMF6 (Filipits et al., 1993) essentially as described for pAG244 (Gurvitz et al., 1997b).

### Table 1. Saccharomyces cerevisiae strains used

| Strains | Description | Sources or references |
|---------|-------------|-----------------------|
| (1) BJ1991 | MATa leu2 ura3-52 trp1 pep4-3 ptr1-1-222 gal2 | Jones (1977) |
| (2) BY991pip2Δ^1 | pip2 Δ:: KanMX4 | Rottensteiner et al. (1997) |
| (3) BY991oaf1Δ^1 | oaf1 Δ:: LEU2 | Rottensteiner et al. (1997) |
| yHPR11550^ | pSP519 ORE: CYC1-lacZ | This study |
| yAG2595^ | pAG244 (PS19 ORE:: CYC1-lacZ) | Gurvitz et al. (1997b) |
| yAG5617^ | pAG534 (PS18-lacZ WT) | Gurvitz et al. (1997b) |
| yAG573^ | pAG528 (SPS18-lacZ M1) | This study |
| yAG554^ | pAG530 (SPS18-lacZ M3) | This study |
| yAG565^ | pAG536 (SPS18-lacZ M3) | This study |
| yAG557^ | pAG532 (SPS18-lacZ M4) | This study |
| yAG569^ | pAG538 (SPS18-lacZ M5) | This study |
| (4) BY991pip2Δoaf1Δ^1 | pip2 Δ:: KanMX4 oaf1 Δ:: LEU2 | Rottensteiner et al. (1997) |
| yAG1310^4 | pAG534 (SPS18-lacZ WT) | This study |
| yAG1312^4 | pAG536 (SPS18-lacZ M3) | This study |
| (5) BY4741 | MATa his3a leu2Δ0 met15Δ0 ura3Δ0 | EUROSCARF |
| (6) BY4741sum1Δ^5 | YDR310c:: KanMX4 | EUROSCARF |
| BY4741his1Δ^6 | YOR068c:: KanMX4 | EUROSCARF |
| BY4741rm1Δ^6 | YOR29c:: KanMX4 | EUROSCARF |
| yAG1193^5 | expressing SPS18-lacZ from pAG534 | This study |
| yAG1230^6 | expressing SPS18-lacZ from pAG534 | This study |
| BY4741oaf1Δ^5 | oaf1 Δ:: LEU2 from pAK83 | This study |
| BY4741pip2Δ^6 | pip2 Δ:: LEU2 from pSKAPIP2 | This study |
| BY4741sum1oaf1Δ^6 | oaf1 Δ:: LEU2 from pSKAPIP2 | This study |
| BY4741sum1pip2Δ^6 | pip2 Δ:: LEU2 from pSKAPIP2 | This study |

*The numbers in superscript following the strains’ designation refer to their parental genotypes; for example, BJ1991pip2Δ^1 was derived from (1) BJ1991.

### Plasmid constructions

Nucleic acids were manipulated as described (Sambrook et al., 1989). Construction of integrative plasmids with promoters containing deletions was described previously (Gurvitz et al., 1997b). Briefly, a 1.4-kb Xba–Splh fragment containing the intergenic region and part of the coding regions of SPS19 was excised from pUC18-KXC (Coe et al., 1994) and inserted into M13mp19 for either deletion of the promoter regions M3, M4, and M5, or substitution at M1 and M3 with a unique Xhol site, using site-directed mutagenesis. The mutated DNA was verified by nucleotide sequencing. Construction of plasmid pAG534 containing the WT SPS18 promoter fused with the lacZ gene in YIp356R (Myers et al., 1986) was outlined previously (Gurvitz et al., 1997b). Plasmids pAG528, pAG530, and pAG532 consisted of the respective M1, M2, and M4 mutated promoters. Plasmids pAG536 and pAG538 (M3 and M5 mutated promoters, respectively) were constructed here. Plasmid pPS19 ORE: CYC1-lacZ was constructed from pMF6 (Filipits et al., 1993) essentially as described for pAG244 (Gurvitz et al., 1997b).

### Table 2. Plasmids and oligonucleotides used

| Plasmids | Description | Sources or references |
|----------|-------------|-----------------------|
| pPS19ORE-SPS19 | SPS19 ORE:: CYC1-lacZ, fusion boundary towards SPS19 | This study |
| pAG244 | SPS19 ORE:: CYC1-lacZ, fusion boundary towards SPS18 | Gurvitz et al. (1997b) |
| pMF6 | Integrative plasmid vector for the above two reporter genes | Filipits et al. (1993) |
| YIp356R/YIp357 | URA3-marked integrative vectors for lacZ fusions | Myers et al. (1986) |
| pAG534 | SPS18–lacZ, YIp356R with 1.4-kb SPS18/lacZ fragment | (1997b) |
| pAG528 | As above but with an Xhol-site substitution at M1 | A. Gallagher, UNSW |
| pAG530 | As above but with a deletion at M2 | A. Gallagher, UNSW |
| pAG536 | As above but with an Xhol-site substitution at M3 | This study |
| pAG532 | As above but with a deletion at M4 | A. Gallagher, UNSW |
| pAG538 | As above but with a deletion at M5 | This study |
| pSKAPIP2 | pip2 Δ:: LEU2 disruption plasmid | Rottensteiner et al. (1996, 1997) |
| pAK83 | oaf1 Δ:: LEU2 disruption plasmid | Rottensteiner et al. (1997) |

| Oligonucleotides | Description | Sources or references |
|-----------------|-------------|-----------------------|
| ACT1-928F | GCGGAAAGATGCAAAAGGA | This study |
| ACT1-1001R | TCTGGAGGAGCAATGACTTGA | This study |
| SMK1-988F | CAAGCTATATCAC | This study |
| SMK1-1060R | AAGGACCCTGAAGGCAAACA | This study |
| ACTCAATTCCTAAC | AAGGACCCTGAAAGGCAAACA | This study |
| SPS18-890F | ATCCAGAGATCATTCGTGCACTTTA | This study |
| SPS18-862R | AAGAAAAAACTGGCGAGGGTAA | This study |
| SPS19-391F | GCCGGTCTGCTGGA | This study |
| SPS19-399R | AACACAGATTTGAGGCGGTTG | This study |
Media and growth conditions

Standard yeast (Rose et al., 1990) and E. coli (Sambrook et al., 1989) media were made as described. *Saccharomyces cerevisiae* strains were propagated on solid rich-glucose YPD medium consisting of 1% (w/v) yeast extract – 2% (w/v) peptone (YP), 2% (w/v) d-glucose, and 2% (w/v) agar. Selection for integrative or disruption plasmids in transformed strains was carried out using solid synthetic defined (SD) medium consisting of 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) d-glucose, 3% (w/v) agar, with all supplements added except for uracil (SD-Ura) or leucine (SD-Leu).

Liquid oleic acid medium (YPO) consisted of YP, 0.05% (w/v) glucose, 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80, adjusted to pH 7 with NaOH (Gurvitz et al., 1997b). For β-galactosidase measurements using o-nitrophenyl-β-D-galactopyranoside (ONPG) (Miller, 1972; Rottensteiner et al., 1996), cells were induced in YPO medium as follows: late log-phase cells from overnight YPD precultures were transferred to 100-mL conical flasks containing 50 mL YPO (with 75 μg mL⁻¹ ampicillin) to A₆₀₀nm = 0.2. The cultures were returned to shaking and samples were removed for analysis at the indicated times. Protein concentrations were determined using the BioRad dye (Bradford, 1976).

RNA isolation

Triplicate cultures of *S. cerevisiae* cells induced in YPO were collected by centrifugation (3000 g at 4 °C for 5 min), washed twice in two volumes of cold distilled water, and frozen in liquid nitrogen. RNA samples were extracted with the Master Pure™ Yeast RNA Purification Kit (Epigen Ingredients, WI) according to the manufacturer’s instructions. Following isolation, RNA was treated twice with an RNase-Free DNase set (Qiagen, Hilden, Germany) in keeping with supplier instructions. To verify the removal of contaminating genomic DNA, RNA samples were subjected to thermocycling amplification without reverse transcriptase.

Quantitative real-time PCR and data analysis

Total RNA (5 μg) was processed using reverse transcriptase into first-strand cDNA in 20-μL reactions with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Helsinki, Finland). To generate primers for real-time PCR, the nucleotide sequences of the *S. cerevisiae* genes *SPS18*, *SPS19*, *SMK1*, and *ACT1* were scrutinized using the Primer Express software (Applied Biosystems, Foster City, CA), and the oligonucleotides were supplied by Sigma-Aldrich Inc. in the United Kingdom. Real-time PCR was performed with an ABI PRISM 7000 sequence detector and analysed using the ABI PRISM 7500 sequence detector software v. 1.4 (Applied Biosystems). Amplification was carried out in 30-μL reaction mixtures consisting of 1 × SYBR Green PCR master mix (Applied Biosystems), 4.5 nL of cDNA reaction mixture and 2 pmol μL⁻¹ primer sets. Thermocycling was performed in 40 cycles of a two-step PCR (95 °C for 15 s and 60 °C for 1 min) after an initial activation (95 °C for 10 min) of DNA polymerase. A heat dissociation protocol was applied to the PCR reactions to ensure that the SYBR green dye detected only one PCR product. Triplicate cDNAs from each sample were amplified using primers for *SPS18*, *SPS19*, *SMK1*, and *ACT1* genes. Two independent assays with the same cDNA samples and primers for *SPS18*, *SPS19*, *SMK1*, and *ACT1* were undertaken and values were measured for each individual experiment. Following SYBR Green PCR amplification, data acquisition and subsequent data analyses were carried out using the ABI PRISM 7500 sequence detector software 1.4.

The PCR cycle at which a statistically significant increase in the ΔRₙ (the fluorescence of SYBR Green relative to that of internal passive dye, ROX) is first detected is called the threshold cycle (Cₘ). The ΔCₘ refers to the difference between the mean Cₘ value of the *SPS18*, *SPS19*, *SMK1*, and the endogenous control, *ACT1*. The ΔΔCₘ represents the difference between the mean ΔCₘ value of the calibrator BY4741 WT culture and the corresponding mutant strains (Table 1). The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by 2⁻ΔΔCₘ. Derivation of the 2⁻ΔΔCₘ equation has been described in Applied Biosystems, User Bulletin No. 2 (P/N 4303859). Hence, experimental samples could be expressed as an n-fold difference relative to the calibrator. For the real-time assays with the 2⁻ΔΔCₘ method, the amplification efficiency of the target gene and internal control gene was tested by plotting the amount of the input template vs. the ΔCₘ, where a slope of c. 0 demonstrates that the efficiencies were comparable.

Results

**SPS19 ORE mediates bidirectional transcription**

Cells propagated in oleic acid medium do not transcribe *SPS18* to the same level as the ORE-dependent gene *SPS19* (Gurvitz et al., 1997a, b). *SPS19* ORE complies with the consensus sequence CGGN₄TN₉/₁₆N₈₋₁₂CCG (Gurvitz & Rottensteiner, 2006), which binds the Pip2p-Oaf1p transcription factor (Luo et al., 1996; Rottensteiner et al., 1996). Although the OREs in the promoters of the *PTT1/FOX3* and *CTA1* genes (Einerhand et al., 1993; Filipits et al., 1993) have been shown to confer bidirectional transcription on a basal *CYC1* promoter, it was not clear from the outset whether the *SPS19* ORE acts equally in both directions.

To examine whether *SPS19* ORE intrinsically activates transcription with a preference towards *SPS19*, the element was tested for orientation bias in conferring transcription on a basal *CYC1* promoter. Cells expressing a *CYC1-lacZ* reporter gene in which *SPS19* ORE was inserted in either orientation
were monitored following 18-h growth on oleic acid. The results demonstrated similar levels of β-galactosidase activity irrespective of insert orientation (c. 20-fold greater than at 0 h; Table 3). This indicated that the minimal sequence of SPS19 ORE did not contain additional information relating to orientation of transcription, albeit nucleotides within the ORE might act in conjunction with neighbouring sequences to effect unidirectionality, such as in the situation with UAS<sub>SPS19</sub> (Gurvitz et al., 1999), in which a mild degree of direction is enforced on the 5′-ORE half site. Therefore, a further stricture or boundary mechanism must exist that confines the regulatory action of the oleic acid-specific trans-activator Pip2p-Oaf1p to transcribing SPS19.

Loss of SPS18 repression

To analyse the SPS18–SPS19 intergenic region for sequences that might be involved in throttling the transcription of SPS18 during oleic acid induction, a set of strains was generated harbouring lacZ reporter genes carrying the WT promoter or a promoter containing deleted segments (M1 through M5; Fig. 1). Levels of β-galactosidase expression by SPS18–lacZ were measured in soluble protein extracts from cells grown overnight on rich-glucose medium (0 h) followed by 18 h propagation on oleic acid. The results demonstrated that the precultures at 0 h with the M1–M4 reporter genes gave rise to levels of β-galactosidase activities that were higher compared with the WT construct (Table 4), although these values were at the lower detection limit of the method used. Following 18-h growth on oleic acid medium, a decreased level of β-galactosidase activity was recorded for the M1 reporter gene compared with the WT, which coincided with perturbed ORE and UAS<sub>SPS19</sub> elements in the promoter of the mutant construct (Fig. 1; Table 4). On the other hand, mutant reporter genes M2–M5 gave rise to activities that were at least twofold higher compared with those obtained using the WT construct (Table 4). This indicated the loss of a potential repressor element (an operator site). For comparison, a previous experiment conducted on the same set of mutations, but in the settings of an SPS19–lacZ reporter, gave the following values: WT, 1 (relative level); M1, 0.01; M2, 0.29; M3, 0.85; M4, 0.92, and M5, 0.67 (Gurvitz et al., 1999). The M3–M4 demarcated region overlaps an MSE (Ozsarac et al., 1997) that could turn out to be a repressor element of the SMK1-NHP6A type (Xie et al., 1999). Hence, loss of the MSE repressor element could lead to (1) a more active basal promoter, (2) a misdirection of ORE-dependent transcriptional activation towards SPS18, or (3) a combination of both. Were the observed unscheduled transcription of SPS18 shown to be subordinate to Pip2p-Oaf1p, this could help elucidate the cause of this effect.

Table 3. The effect of inserting SPS19 ORE in either orientation on the transcription of an integrative basal CYC1–lacZ reporter construct in haploid cells following oleic acid induction

| Strains   | Direction of lacZ fusion | β-Galactosidase activity* | Fold induction Relative level |
|-----------|--------------------------|---------------------------|------------------------------|
| yHPR1550  | Towards SPS19             | 11                        | 3.8 x 1.0                    |
| yAG259    | Towards SPS18             | 7                         | 1.0                          |

*nmol ONPG metabolized min<sup>−1</sup> mg<sup>−1</sup> protein.

Table 4. The effect of deletions in the SPS18–SPS19 promoter on the expression of an integrative SPS18–lacZ reporter gene in haploid cells grown under oleic acid medium conditions

| Strains   | Reporters Mutated at     | β-Galactosidase activity* | Fold induction Relative level |
|-----------|--------------------------|---------------------------|------------------------------|
| yAG561    | WT Not mutated           | 15 ± 1                    | 3.8 x 1.0                    |
| yAG547    | M1 ORE, UAS<sub>SPS19</sub> | 8 ± 3                    | 1.6 x 0.6                    |
| yAG554    | M2 UAS<sub>SPS19</sub>   | 42 ± 6                    | 4.2 x 2.8                    |
| yAG565    | M3 SPS18 M5             | 38 ± 6                    | 7.6 x 2.6                    |
| yAG557    | M4 SPS18 M5             | 43 ± 8                    | 6.1 x 2.9                    |
| yAG569    | M5 3′ to MSE            | 31 ± 12                   | 10.3 x 2.0                   |

*nmol ONPG metabolized min<sup>−1</sup> mg<sup>−1</sup> protein.

SPS18 MSE represses SPS18 transcription on glucose and oleic acid

The SMK1-NHP6A MSE blocks the expression of the former gene from the constitutive transcription of the latter, thereby representing a repressor element (Xie et al., 1999). In the case of SPS18, however, the divergent SPS18 gene is not constitutively expressed, but instead is subordinate to an ORE that induces transcription by over 20-fold in response to oleic acid (Gurvitz et al., 1997a, b). The SPS19 ORE palindrome is overlapped by other elements at each half site, an Adr1p-binding element UAS<sub>SPS19</sub> at its 3′-half site, and a separate UAS<sub>SPS19</sub> at the other. SPS19 transcription fails to become induced in the absence of either the Pip2p-Oaf1p or Adr1p transcription factors that have been shown to bind SPS19 ORE and UAS<sub>SPS19</sub>, respectively.

If loss of the MSE leads to higher basal activity of SPS18, then we would predict that the level of transcription would not depend on the carbon-source responsiveness of the Pip2p-Oaf1p activator complex. On the other hand, were the SPS18 MSE to shield SPS18 in cells grown on oleic acid from the high levels of ORE-dependent transcriptional activation of SPS19, unscheduled transcription of SPS18 would be subordinate to Pip2p-Oaf1p. To examine which of the two possible scenarios predominates, the previously used SPS18–lacZ reporter genes WT and the MSE-less M3 were examined in WT cells as well as a pip2Δoaf1Δ mutant.
strain (Karpichev et al., 1997; Rottensteiner et al., 1997) in which transcriptional activation of SPS19 is abrogated (Gurvitz et al., 1997a). Both reporter genes contain an intact ORE; Pip2p-Oaf1p is not known to interact with MSEs.

The results demonstrated that levels of β-galactosidase activities expressed from the M3-reporter gene in the WT strain yAG565 following overnight growth in rich-glucose medium (0 h) were slightly higher than from the WT reporter in the WT strain yAG561 grown under similar conditions (3 U compared with 2 U; Table 5). Notwithstanding the fact that these 0-h measurements of the precultures were at the detection limit, this indicated that the basal activity of the promoter might have been higher in the reporter construct lacking a complete MSE. Following 18-h oleic acid-medium conditions, β-galactosidase activities from the M3 fusion in yAG565 were c. 2.5-fold higher than those from the WT fusion in yAG561 (27 U compared with 11 U; Table 5), and almost twofold higher compared with the pip2Δoaf1Δ mutant yAG1312 (27 U compared with 15 U; Table 5), whereas expression levels of the parental WT reporter gene in both the WT strain yAG561 as well as in the pip2Δoaf1Δ mutant yAG1310 were essentially identical, at about 11 U. Hence, from the results presented here, it emerged that in addition to repressing SPS18 transcription under vegetative conditions, the MSE also appeared to play a role in shielding SPS18 from unscheduled ORE-dependent transcription (Fig. 2). Confirmation of these reporter-gene results was undertaken using quantitative real-time PCR.

### SPS18 MSE relies on Sum1p for repressing SPS18

MSEs represent the target for the sporulation-specific transcription factor Ndt80p (Xu et al., 1995; Chu et al., 1998). In addition, MSEs are also the target for Sum1p – in association with Hst1p and Rfm1p (Xie et al., 1999; McCord et al., 2003), which act in unison to repress certain midsporulation genes under vegetative conditions (Xie et al., 1999). To determine whether Sum1p is important for repressing SPS18, a single copy of the WT SPS18–lacZ reporter gene was introduced into the genome of a WT BY4741 haploid

**Table 5.** The effect of deleting PIP2 and OAF1 on the expression of WT and M3 SPS18–lacZ reporter genes in haploid cells grown under oleic acid-medium conditions

| Strains | β-Galactosidase activity* | Fold induction level |
|---------|--------------------------|---------------------|
|         | 0 h | 18 h |                      |
| WT reporter (intact MSE) | | | |
| A  yAG561 WT | 2 | 11 | 5.5 x 1.0 |
| B  yAG1310 pip2Δoaf1Δ | 1 | 11 | 11.0 x 1.0 |
| M3 reporter (mutated MSE) | | | |
| C  yAG565 WT | 3 | 27 ± 1 | 9.0 x 2.5 |
| D  yAG1312 pip2Δoaf1Δ | 3 | 15 ± 1 | 5.0 x 1.3 |

*nmol ONPG metabolized min⁻¹ mg⁻¹ protein.

1 Mean ± SD; n = 3.

2 Refers to the element arrangement in Fig. 2.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Scheme of the promoter arrangement in the strains reported in Table 5. In the WT strain yAG561 expressing the WT reporter gene (a), Sum1p acting at the MSE is proposed to block oleic acid-induced transcriptional activation due to Pip2p-Oaf1p binding to the ORE, so that no decrease in reporter-gene expression levels was detected when Pip2p-Oaf1p was absent in the mutant strain yAG1310 (b). However, when the MSE was mutated within the M3 reporter construct (c) and Sum1p could not act on its cognate element, Pip2p-Oaf1p could induce transcription of SPS18 (d) in the WT strain yAG565 beyond the levels attained by the pip2Δoaf1Δ mutant yAG1312 (d). Thick and thin arrows indicate high or low levels of transcription, respectively. UAS refers to an overlapping Adr1p-binding element.
strain as well as into that of an otherwise isogenic mutant with a deletion in the SUM1 gene.

The results of the reporter-gene assays performed on these two strains (Table 6) showed that following an 18-h propagation on oleic acid medium, β-galactosidase activities in the WT harbouring SPS18–lacZ increased 1.6-fold compared with glucose, whereas in the sum1Δ mutant this increase was 3.7-fold. On oleic acid, SPS18–lacZ was 2.9-fold more highly expressed in the sum1Δ mutant than in the WT (116 vs. 40 U) as compared with a 1.2-fold increase in activity between these two strains when grown on glucose (31 vs. 25 U). Hence, Sum1p appeared to shield SPS18 from oleic acid-induced transcription activation (Fig. 3), and this could be exposed using quantitative real-time PCR.

**Quantitative real-time PCR of the roles of Sum1p/Hst1p/Rfm1p in repressing SPS18**

To determine the physiological levels of SPS18 transcripts when the MSE is not occupied by Sum1p or its two associates, quantitative real-time PCR was performed. Yeast cells were propagated in liquid oleic acid medium for 18 h, and following cell breakage, RNA was extracted (with the concomitant removal of contaminating DNA) in order to provide template for thermocycling amplification, which was applied to SPS18

**Table 6.** The effect of mutating SUM1 on SPS18–lacZ reporter gene expression following an 18-h oleic acid induction of haploid cells

| Strains | β-Galactosidase activity* | Fold induction | Relative level |
|---------|--------------------------|----------------|---------------|
| A yAG1230 WT 25 40 ± 3 1.6 x 1.0 |
| B yAG1193 sum1Δ 31 116 ± 5 3.7 x 2.9 |

*nmol ONPG metabolized min⁻¹ mg⁻¹ protein.

1 Mean ± SD; n = 3.

2 Refers to the element arrangement in Fig. 3.

Table 7. Real-time PCR revealing the effect of deleting SUM1, HST1, or RFM1 on SPS19 and SPS18 expression in haploid cells following an 18-h oleic acid induction

| Strains | Genes | Average C<sub>T</sub> | Average ΔΔC<sub>T</sub> ± SE | ΔΔC<sub>T</sub> | RQ |
|---------|-------|----------------------|-----------------------------|--------------|-----|
| WT SPS18 | 33.120 | 11.089 ± 0.222 | 0 | 1 |
| SPS19 | 29.388 | 1.357 ± 0.510 | 0 | 1 |
| SMK1 | 29.961 | 7.930 ± 0.243 | 0 | 1 |
| ACT1 | 22.031 | |
| sum1Δ SPS18 | 29.855 | 7.831 ± 0.234 | -3.258 | 9.564 |
| SPS19 | 22.282 | 0.258 ± 0.267 | -1.099 | 1.142 |
| SMK1 | 29.275 | 7.252 ± 0.267 | -0.678 | 1.160 |
| ACT1 | 22.024 | |
| hst1Δ SPS18 | 30.620 | 8.272 ± 0.039 | -2.817 | 7.047 |
| SPS19 | 22.673 | 0.325 ± 0.046 | -1.032 | 2.045 |
| SMK1 | 28.748 | 6.401 ± 0.114 | -1.529 | 2.887 |
| ACT1 | 22.348 | |
| rfm1Δ SPS18 | 31.176 | 8.120 ± 0.234 | -2.969 | 7.829 |
| SPS19 | 23.080 | 0.025 ± 0.038 | -1.333 | 2.519 |
| SMK1 | 29.263 | 6.208 ± 0.158 | -1.722 | 3.300 |
| ACT1 | 23.056 | |

*In this and the ensuing Tables 8 and 9, the significance of C<sub>T</sub>, AC<sub>T</sub>, and ΔΔC<sub>T</sub> is explained in Materials and methods.

RQ, relative quantity.

Fig. 3. Scheme of the promoter arrangement in the strains reported in Table 6. In the WT strain yAG1230 expressing the WT SPS18–lacZ reporter construct (a), Sum1p could act at the MSE to block oleic acid-induced transcriptional activation (→) due to Pip2p-Oaf1p binding to the ORE, but in yAG1193 cells devoid of Sum1p (b); this latter activation proceeded unhindered (←), resulting in higher levels of reporter-gene activity. The thick arrows reflect higher levels of transcription compared with those depicted by the thin arrows. The Adr1p-binding element overlapping the ORE is referred to as UAS.
on elevating SPS19 expression in these mutants was also noted (2.1–2.5-fold).

To determine whether the increase in SPS18 expression in the sum1Δ mutant was due, at least in part, to oleic acid-dependent induction, sum1Δoaf1Δ and sum1Δpip2Δ double mutants were generated and examined for their ability to express SPS18 and SPS19. The rationale behind this experiment was that if SPS18 expression in the double mutants was lower than in the parental sum1Δ mutant (as would be expected for the Pip2p- and Oaf1p-dependent gene SPS19), this would indicate that in sum1Δ cells loss of MSE function allowed oleic acid-dependent transcription to proceed in the wrong orientation.

The results in Table 8 showed that in the WT strain, SPS19 amplification occurred considerably earlier compared with SPS18. As expected, deletion of OAF1 in the formerly WT strain resulted in a dramatic 42-fold reduction in SPS18 expression, validating the phenotype of the mutant generated with the disruption plasmid intended for subsequent integration in the sum1Δ strain. Interestingly, this deletant expressed SPS18 eightfold less efficiently than did the WT. Although the unleashing effect of the sum1Δ deletion on SPS18 expression was not as high in the present experiment compared with the situation in Table 7, nevertheless expression of SPS18, SMK1, and SPS19 was increased. Importantly, deletion of OAF1 in the sum1Δ strain, which was validated by the observation of a sevenfold reduction in SPS19 expression compared with the WT situation, ended up cancelling the derepressing effect of the sum1Δ mutation on SPS18.

To confirm these latter results, a further experiment was carried out, in which the cumulative effect of a pip2Δ deletion on top of that of sum1Δ was examined (Table 9).

| Strains | Genes | Average C<sub>i</sub> | Average ΔC<sub>i</sub> ± SE | ΔΔC<sub>i</sub> | RQ |
|---------|-------|----------------------|-----------------------------|-------------|-----|
| WT      | SPS18 | 28.701               | 6.369 ± 0.415              | 0           | 1   |
|         | SPS19 | 22.432               | 0.101 ± 0.425              | 0           | 1   |
|         | SMK1  | 30.164               | 7.832 ± 0.39               | 0           | 1   |
|         | ACT1  | 22.331               |                             |             |     |
| oaf1Δ   | SPS18 | 31.573               | 9.419 ± 0.383              | 3.049       | 0.121|
|         | SPS19 | 27.651               | 5.497 ± 0.121              | 5.396       | 0.024|
|         | SMK1  | 30.1                | 7.946 ± 0.293              | 0.114       | 0.924|
|         | ACT1  | 22.154               |                             |             |     |
| sum1Δ   | SPS18 | 28.908               | 4.118 ± 0.916              | –2.251      | 4.762|
|         | SPS19 | 24.793               | 0.003 ± 0.976              | –0.098      | 1.07 |
|         | SMK1  | 31.873               | 7.083 ± 0.985              | –0.749      | 1.681|
|         | ACT1  | 24.79                |                             |             |     |
| sum1Δoaf1Δ | SPS18 | 29.109               | 6.552 ± 0.499              | 0.183       | 0.881|
|         | SPS19 | 25.45                | 2.893 ± 0.655              | 2.793       | 0.144|
|         | SMK1  | 29.943               | 7.386 ± 0.408              | –0.446      | 1.362|
|         | ACT1  | 22.556               |                             |             |     |

RQ, relative quantity.

In this round of oleic acid induction, SPS19 was almost 1400-fold more highly expressed compared with SPS18. In the corresponding pip2Δ mutant generated here, SPS19 was almost tenfold less efficiently expressed compared with the WT, verifying the mutant’s phenotype for reduced expression of ORE-regulated genes as a result of the integration of the disruption plasmid. Like the above situation with the oaf1Δ deletion, SPS18 expression in the pip2Δ mutant was also affected, albeit to a lesser extent than SPS19. Introduction of the pip2Δ deletion into the sum1Δ mutant resulted in an almost fourfold reduced efficiency in SPS19 expression by the double deletant compared with the sum1Δ deletion alone, thereby authenticating the former’s mutant phenotype. The observation made here with sum1Δpip2Δ cells, which duplicated that made with the previous sum1Δoaf1Δ strain in exposing the overall reduction in SPS18 expression as a result of altering the oleic acid-induction machinery in the sum1Δ mutant, is discussed.

**Discussion**

Here, we revealed an important part of the mechanism in *S. cerevisiae* for repressing the sporulation-specific gene SPS18 under vegetative conditions, and for shielding it from unscheduled transcriptional activation in haploid cells grown on oleic acid. Induction of the divergent partner SPS19 is instigated by Pip2p-Oaf1p and Adr1p acting at the divergent and promoter regions of SPS19, respectively. The repression element of SPS18 was shown here to be comprised of an MSE, but
might also involve neighbouring elements including a unidirectionally acting enhancer element UAS\(^{\text{SPS19}}\) and the M5 element (Fig. 1).

Ndt80p-binding MSEs have the consensus sequence YGNCRCAA\(^{Ay/p}\) and act to upregulate some 300 genes in sporulating diploid cells midway through meiosis (Hepworth et al., 1995, 1998; Ozsarac et al., 1997; Chu et al., 1998). The MSEs in the promoters of the SMKI, NDT80, and SPR3 genes are additionally targeted by Sum1p in association with Hst1p and Rfm1p to repress the corresponding genes in vegetative cells (McCord et al., 2003; Pierce et al., 2003; Xie et al., 1999). The nucleotide sequence of the MSE in the shared promoter region of SPS18 and SPS19 is very close agreement with the consensus for a Sum1p-binding MSE AGYGWCACAAAAD, with a tolerable G to A deviation at the noncritical position 2 (Pierce et al., 2003). From the findings presented here, the SPS18 MSE is a repressing element in vegetative cells grown on glucose medium. SPS18 MSE additionally maintains blockage of SPS18 transcription under fatty acid-medium conditions, when transcription of SPS19 is highly active. This was manifested in the situation with the MSE-less version of SPS18–\(\beta\)-gal (M3) whose expression was higher in oleic acid-grown WT cells compared with pip2\(\Delta oaf1\)\(\Delta\) mutants, in which the response to oleic acid was impaired, and reinforced by the lowered SPS18 expression seen in both sum1\(\Delta oaf1\)\(\Delta\) and sum1\(\Delta pip2\)\(\Delta\) deletants as compared with the situation in the parental sum1\(\Delta\) mutant.

Two previous studies place SPS18 high on the list of oleic acid-induced genes (Koerkamp et al., 2002; Smith et al., 2002). In one case (Smith et al., 2002), use of diploid cells grown on glycerol before being shifted to oleic acid medium probably introduced an additional physiological response associated with starving cells being synchronized for meiosis and sporulation. This could explain the appearance of SPS18 in the list of oleic acid-inducible genes, because its expression is a clear indication for onset of sporulation-specific processes (Coe et al., 1994; Chu et al., 1998). The explanation for why SPS18 appears on a separate list of highly inducible genes using haploid cells (Koerkamp et al., 2002) rests on the issue of whether the DBY7286 strain used is really WT for expressing SPS18. Because DBY7286 is a descendant of S288c (as is the EUROSCARF strain BY4741 used here) that harbours a mutation in the gene for the Hap1p transcription factor, it is modified in many aspects of its respiratory and oxygen metabolism (Gaisne et al., 1999). At least in our hands, real-time amplification of SPS18 considerably lagged that of SPS19 in all the experiments.

It is tempting to view the mechanism by which transcriptional regulation of one gene is blocked from affecting that of its diverging gene as a form of insulation. Indeed, insulators in higher eukaryotes are defined in part as elements with the ability to block transcriptional activation of a promoter by a nearby enhancer (Bi & Broach, 1999). The second insulator criterion, to protect transgenes from positive or negative position effects (Bi & Broach, 1999), was not relevant to the present work. However, caution is urged before reclassifying a yeast repressing sequence as an insulator, because at this point it is not clear whether the two mechanisms of action are identical. Nevertheless, it is hoped that the resemblance between the end effects orchestrated by these two mechanisms of shielding genes from unscheduled transcription will spur additional studies into this rather underappreciated phenomenon in yeast.

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Authors’ contribution
A.G., F.S. and H.R. contributed equally to this paper.

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