The product of the mei3\textsuperscript{+} gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast

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In fission yeast the ability to undergo meiosis and sporulation is conferred by the matP\textsuperscript{+} and matM\textsuperscript{+} genes of the mating-type locus. Inactivation of ran1\textsuperscript{+}, a negative regulator of meiosis, is thought to be an essential step in meiotic initiation. We have isolated a further meiotic control gene mei3\textsuperscript{+}, and have shown the following: a null allele of mei3 totally inhibits meiosis; the mei3\textsuperscript{+} RNA transcript and its translational product are expressed only in matP\textsuperscript{+}/matM\textsuperscript{+} diploids entering meiosis; forced expression of mei3\textsuperscript{+} in vegetative cells provokes haploid meiosis and sporulation. We suggest that the product of mei3\textsuperscript{+} gene, a protein of 21 kd, initiates meiosis by inactivating ran1\textsuperscript{+}.

Key words: Schizosaccharomyces pombe/meiosis/mei3

Introduction

The fission yeast, Schizosaccharomyces pombe, has two primary cell types known as h\textsuperscript{+} and h\textsuperscript{−} (Leupold, 1950). They differ only in the allele expressed at the mating-type locus, matP\textsuperscript{+} in an h\textsuperscript{+}, and matM\textsuperscript{+} in an h\textsuperscript{−} cell (Leupold, 1958). In wild-type strains (h\textsuperscript{h0}; homothallic, 90\% self-mating) matP\textsuperscript{+} and matM\textsuperscript{+} interconvert every few cell generations by a unidirectional gene conversion or ‘cassette’ mechanism (Hicks et al., 1977; Beach, 1983; Egel, 1984).

The mating type of the fission yeast is not apparent unless the culture medium becomes limited for certain nutrients (Egel, 1971). In response to starvation, h\textsuperscript{+} and h\textsuperscript{−} cells fuse with each other and thereby form a third cell type, the h\textsuperscript{+}/h\textsuperscript{−} diploid zygote. Under certain conditions the h\textsuperscript{+}/h\textsuperscript{−} zygote can be propagated vegetatively but upon starvation it initiates meiosis and sporulation (Egel and Egel-Mitani, 1974). Cells that are committed to meiosis undergo pre-meiotic DNA replication (Egel and Egel-Mitani, 1974; Beach et al., 1985), which is followed closely by the reductional and equational meiotic divisions. Each of the four resulting haploid meiotic nuclei is packaged into a highly resistant ascospore (Leupold, 1950).

Although cell type (h\textsuperscript{+}, h\textsuperscript{−} or h\textsuperscript{+}/h\textsuperscript{−}) is determined by the matP\textsuperscript{+} and matM\textsuperscript{+} genes of the mating-type locus, expression of the sexually differentiated state appears to be controlled by the ran1\textsuperscript{+} gene (referred to as pat1\textsuperscript{+} by some authors), which is a negative regulator of both conjugation and sporulation (Nurse, 1985; Iino and Yamamoto, 1985a; Beach et al., 1985). Strains carrying a recessive temperature-sensitive allele of ran1 have a remarkable phenotype. After transfer from a permissive to a semi-permissive temperature, actively dividing cells in early log-phase culture slow their rate of growth, accumulate in the G\textsubscript{1} phase of the cell cycle, conjugate with cells of opposite mating type and finally sporulate (Nurse, 1985; Beach et al., 1985). The normal requirement of starvation for the initiation of conjugation and sporulation is bypassed.

Complete inactivation of ran1\textsuperscript{+} activity, following abrupt transfer of a non-leaky ran1ts mutant strain to a fully restrictive temperature has a more dramatic effect. Vegetative growth and cell division are totally inhibited (Beach et al., 1985) and haploid cells sporulate directly without conjugating (Nurse, 1985; Iino and Yamamoto, 1985a,b; Beach et al., 1985). Both normal preconditions for meiosis and sporulation, heterozygosity at the mating-type locus (matP\textsuperscript{+}/matM\textsuperscript{+}) and nutritional starvation, are rendered inessential following total loss of ran1\textsuperscript{+}. Since the events which occur in haploid cells following inactivation of ran1\textsuperscript{+} bear many similarities to true meiosis (Iino and Yamamoto; 1985b, Beach et al., 1985) it has been proposed that inactivation of ran1\textsuperscript{+}, under the control matP\textsuperscript{+} and matM\textsuperscript{+}, is the key step which triggers meiotic initiation in h\textsuperscript{+}/h\textsuperscript{−} zygotes (Iino and Yamamoto, 1985a; Beach et al., 1985; Beach, 1985, Nurse, 1985).

At least one of the products of the MAT genes of the distantly related yeast, Saccharomyces cerevisiae, is a DNA binding protein which controls the expression of unlinked genes (Johnson and Herskowitz; 1985). It was anticipated, therefore, that ran1\textsuperscript{+} might be transcriptionally regulated by matP\textsuperscript{+} and matM\textsuperscript{+}. However, the ran1\textsuperscript{+} transcript was found to be equally abundant during vegetative growth and meiosis (McLeod and Beach, 1987). Thus, if inactivation of ran1\textsuperscript{+} is indeed an essential step in meiotic initiation, the mating-type genes must influence ran1\textsuperscript{+} indirectly, presumably by controlling the expression of one or more intermediate genes that which in turn regulate the activity of the ran1\textsuperscript{+} gene product. mei3\textsuperscript{+} is a candidate for this role.

In addition to matP\textsuperscript{+} and matM\textsuperscript{+}, mei2\textsuperscript{+} and mei3\textsuperscript{+} are the only presently known genes of fission yeast which are required specifically for meiotic initiation (Bresch et al., 1968). Strains carrying a recessive mutation in either mei2 or mei3 have a superficially identical phenotype. Vegetative growth and sexual conjugation are unaffected but meiosis becomes arrested at a very early step, before the initiation of premeiotic DNA synthesis (Egel and Egel-Mitani, 1974; Beach et al., 1985). However, the mei mutants respond quite differently to loss of ran1\textsuperscript{+}. Whereas a mei3ran1ts double-mutant stops dividing and sporulates at a non-permissive temperature, a mei2ran1ts strain neither ceases vegetative growth nor sporulates (Iino and Yamamoto, 1985b; Beach et al., 1985). Thus lack of mei3\textsuperscript{+} does not interfere with expression of the ran1ts phenotype, whereas loss of mei2\textsuperscript{+} suppresses both the growth defect and meiotic initiation in the ran1ts mutant. These observations suggest that mei3\textsuperscript{+} acts ‘upstream’ and mei2\textsuperscript{+} ‘downstream’ of ran1\textsuperscript{+} in the meiotic pathway.

Since mei3\textsuperscript{+} is normally essential for meiotic initiation, unless ran1\textsuperscript{+} is inactivated by mutation, mei3\textsuperscript{+} could be an intermediate in a pathway which renders the ran1\textsuperscript{+} gene product subject to control by the mating-type genes in h\textsuperscript{+}/h\textsuperscript{−} diploid cells. In this study, we present evidence that supports this idea.

Results

Null-allele of mei3

The mei3\textsuperscript{+} gene was isolated by transformation of a sporulation defective homothallic strain, h\textsuperscript{h0}leu1.32mei3.71 (Spo\textsuperscript{−}, Leu\textsuperscript{−}),
Fig. 1. Restriction map of a 2.6-kb HindIII restriction fragment containing the mei3+ gene. The heavy line indicates the 1952-bp region which was sequenced. An ORF of 148 amino acid residues is marked. The 5' and 3' ends of the transcript of mei3 and an adjacent gene are marked. The end points of deletions at the 5' and 3' ends of mei3 are shown. Each construction was scored as active (+) or inactive (-), according to criteria described in the text. The 5' and 3' deletions were carried in the vectors pART1 (pmei3.15' series) and pUC118 (pmei3.14 series) respectively. pmei3.15'Δ4+1 was derived from pmei3.15'Δ4 by cutting and filling in the unique SpeI site in mei3. A, B and C indicate three single-stranded probes used for S1 transcript mapping (Fig. 4).

to Leu+ with a bank of wild-type fission yeast DNA carried in a yeast/bacterial shuttle vector, pDB248 (Beach and Nurse, 1981; Beach et al., 1982). Eleven Spo+ transformants were identified after exposure to iodine vapour, a treatment which specifically stains spore-containing colonies (Gutz et al., 1974). Plasmids that conferred an unstable Spo+ phenotype were recovered from yeast by transformation of Escherichia coli with DNA prepared from the yeast transformants. Most of the plasmids contained a common 2.6-kb HindIII fragment of yeast DNA which was shown to be sufficient to rescue mei3.71 (Figure 1).

In the course of further subcloning it was found that all DNA fragments which lacked the unique KpnI site within this HindIII restriction fragment resulted in loss of Spo+ activity (Figure 1). The LEU2 gene of S. cerevisiae was inserted into this KpnI site. This construction (mei3::leu) was returned to the chromosome of an h80leu1.32 strain of yeast by one-step gene-replacement (Rothstein, 1983) of the wild-type gene by mei3::leu. Approximately 10% of Leu+ transformants displayed a stable Spo- phenotype. Southern blotting (Southern, 1975) of the genomic DNA of one such transformant confirmed that the wild-type gene had been replaced by the derivative containing the LEU2 insertion (Figure 2B). This strain, h80leu1.32mei3::leu, failed to sporulate after self-conjugation (Figure 2A), or in an out-cross with a mei3.71 strain, but sporulated normally in an out-cross with a mei3+ strain. This experiment demonstrates that we have isolated and disrupted the mei3+ gene. The phenotype of the presumed null-allele of mei3 (mei3::leu) could not be distinguished from that of the original mei3.71 allele (Bresch et al., 1968; Egel, 1973). mei3+ therefore appears to be required for no cellular activity other than meiotic initiation.

The restriction map of our isolate of mei3+ (Figure 1) is in accord with that published by others (Shimoda and Uehira, 1985).
**Structure of mei3**

The nucleotide sequence of a 1952-bp *HindIII/EcoRI* restriction fragment containing the mei3+ gene. The predicted translational product of 148 amino acid residues is indicated. The initiating ATG and the ATG of Met 76 are underlined. The unique SpeI site is marked. The sites of transcriptional initiation and termination are shown respectively by closed and open boxes. The closed and open triangles mark the endpoints of the deletions from the 5' and 3' ends of the gene, shown in Figure 1.

The 5' initiation site of the mei3+ transcript, which is expressed only during meiosis (see below), was mapped to the precise nucleotide (Figures 1.3, 4A). It initiated at two sites, 87 and 86 bp upstream of the potential translational start of the 148a.a ORF. Within the intervening 87-bp region there are no other AUG codons, in any of the three possible reading frames (Figure 3). The transcript was found to span the ORF without interruption (data not shown) and to terminate ~716 nucleotides beyond the end of the region of ORF (Figures 1 and 4B).

The significance of the 148a.a. ORF was investigated further by construction of a series of deletions, which extended into either the 5' or the 3' end of the transcribed region of mei3+ (Figure 1). Unidirectional deletions at the 3' end of the gene were generated by the method of Henikoff (1984) in the bacterial vector pUC18 which is derived from pUC118 (see Materials and methods). pUC118 can be isolated as either double- or single-stranded DNA and therefore allowed direct sequence determination of each deletion breakpoint without the necessity of further subcloning (Figures 1 and 3). Each construction was introduced into an *hmei3+* strain of yeast by co-transformation with the yeast vector pDB248 (Beach and Nurse, 1981). Deletions were classified as mei3+ if 10–30% of the *Leu*+ transformants also displayed an unstable Spo+ phenotype. By this method...
Deletions at the 5' end of the gene were generated by the same method but in a newly constructed yeast expression vector, pART1, which is derived from pUC118 (see Materials and methods). It carries an *S. pombe* *ars* sequence (Losson and Lacroute, 1983), the *LEU2* gene of *S. cerevisiae* (Beggs, 1978) and the constitutively expressed promoter of the fission yeast alcohol dehydrogenase gene (*adh*, Russell, 1983). Transcription of the *mei3* deletions was driven by the *adh* promoter and the experiment therefore assayed the translational and not the transcriptional importance of sequences at the 5' end of the gene. Each construction was assayed not for its ability to rescue *mei3/71* but for its lethality in transformation of a wild-type yeast strain. This was found to be the consequence of expressing *mei3* + in vegetative cells (see below).

Surprisingly, deletions which extended up to and into the initiating AUG of the 148A.a ORF (pmei3.15'D1−D5) retained *mei3* + activity (Figures 1 and 3). However, a +1 frameshift mutation, (pmei3.15'D4+1) created by opening and filling in the 4-bp 5' overhang of a unique *SpeI* site within the ORF, destroyed the activity of an otherwise functional gene. This frameshift construction could transform a *mei3/71* strain to Leu + at high frequency and did not rescue its sporulation defect.

Since the first AUG of the 148A.a ORF provides the only possible methionine codon, in any of the three possible reading frames, between the 5' end of the *mei3* transcript and the frameshift-sensitive *SpeI* site, this codon must be used for translational initiation. It follows that the construction (pmei3.15'D5) which lacks this initiating codon must make use of the only other in-frame AUG at Met 76 and synthesizes a truncated polypeptide. This suggests that the amino-terminal half of the *mei3* + protein is not absolutely essential for its function.

The 148 amino acid ORF is predicted to encode a protein which, in native form, would have a mol. wt of 21 kd. The protein is basic and consists of 15% serine and 15% threonine (Figure 3). It shows no striking homology to any sequence contained in the widely available protein data bases.

In order to confirm that *mei3* + does indeed encode the protein predicted from the nucleotide sequence and *S1* mapping data, the gene was expressed in *E. coli* and monoclonal antibodies were prepared against *mei3* + protein purified from bacteria (see Materials and methods). The presence of a 21-kd protein that cross-reacts with an anti-*mei3* monoclonal antibody was confirmed by western blotting of whole cell lysates of yeast (Figure 5A).

**Meiotic expression of mei3 transcript and gene product**

The gene which lies immediately adjacent to *mei3* + was found to be expressed constitutively under the conditions tested (Figure 4D, E), but the *mei3* + transcript was detectable only in meiotic cells. A non-meiotic strain of yeast (h +h−) contained the transcript neither during vegetative growth nor after being driven into stationary phase by nitrogen source (NH₄Cl) starvation (Figure 4C). Likewise, a strain that was heterozygous at the mating-type locus but that was meiotically defective due to a specific mutation in *matP* (matP102 see Discussion; Egel, 1984; Bresch et al., 1968) did not transcribe the gene (Figure 4C).
A strain which has the potential to sporulate (h+/h−) also failed to express mei3+ both during vegetative growth and after entering asexuall stationary phase (Figures 4C and 6A). However, the transcript was observed in the same strain that was entering meiosis as a result of nitrogen starvation (Figures 4C and 6A) and the appearance of the transcript was accompanied by accumulation of the 21-kd protein product which was absent in nonmeiotic cells (Figure 5B). Since the transcript accumulated at high levels in vegetative cells if its expression was driven by the adh promoter (Figure 4A, B), the absence of the normal transcript except during meiosis suggests that the mei3+ gene is transcribed only in meiotic cells.

In preliminary experiments it was found that expression of the matP1+ mating-type gene was induced by nitrogen starvation. Since matP1+ is essential for mei3+ transcription (Figure 4C) it was of interest to compare the accumulation of the mei3+ and matP1+ transcripts during meiotic initiation. In the experimental protocol employed, an h+/h− diploid strain was propagated vegetatively in a medium which does not allow meiosis. At stationary phase, the cells were transferred to medium lacking both nitrogen and carbon sources (see legend to Figure 6). Meiosis was initiated by addition of 0.1% glucose, 1% glycerol to the nitrogen-free culture, and within 6 h 70% of the cells had become irreversibly committed (Figure 6B, see Figure 7B for appearance of asyzyotic asci). During this period the matP1+ and mei3+ transcripts accumulated synchronously but whereas the level of the mei3+ transcript was stable after 2.5 h, the matP1+ product declined after 4 h (Figure 6A).

Other authors have reported that although the mei+ transcript is most abundant during meiosis, it is also detectable in vegetative cells (Shimoda and Uehira, 1985). This misconception appears to have arisen because the hybridization probe employed (2.6-kb HindIII fragment, Figure 1), spanned not only mei3+ but also part of the adjacent constitutively expressed gene (Figure 1). The transcripts of the two genes differ in size by little more than 50 nucleotides and are not easily resolved by the method of Northern blotting (Figure 4E) which was used (Shimoda and Uehira, 1985). In fact, the mei3+ gene is transcribed only during meiosis and it is probably vital that it is not expressed in vegetative cells (see below).

Vegetative expression of mei3+ phenocopies ranI null-allele

The effects of expressing mei3+ in a vegetative cell have been investigated by introducing the gene into yeast on the expression vector, pART1 (see Materials and methods). The particular construction used, pmei3.15'Δ4, initiates mei3+ transcription within six nucleotides of the 5' end of the natural transcript (Figures 1, 3, and 4A) and allows transcriptional termination at the normal site (Figure 4B). Western blotting revealed that pmei3.15'Δ4 directs expression of the 21-kd mei3+ gene product (Figure 5A). However, the adh promoter drives mei3+ transcription more vigorously than the natural promoter even during meiosis (Figures 4A, B and 5A).

pmei3.15'Δ4 transformed both a wild-type (h−S or h80) and a mei3.7I strain of yeast to Leu+, at a frequency 100- to 1000-fold lower than the same vector lacking the mei3 insert.
This effect apparently requires both transcription and translation of the mei3+ gene, since constructions in which the mei3+ sequence had been either inverted with respect to the adh promoter or subjected to frame-shift mutation (pmei3.15Δ4+1) yielded normal numbers of transformants.

We reasoned that mei3+ might be lethal to a vegetative cell because its product inactivates ran1+, thereby inhibiting growth and provoking haploid sporulation (see Introduction and Discussion). A direct prediction of this hypothesis follows. A strain which carries an extragenic suppressor of a null-allele of ran1 continues to grow vegetatively and does not sporulate following inactivation of ran1. Such a strain should, therefore, be resistant to vegetative expression of mei3+ if inhibition of ran1+ is the only consequence of expressing this gene in a mitotically dividing cell. mei2.16 is a suitable suppressor mutation (Beach et al., 1985; see Introduction) and, in accordance with the prediction, pmei3.15Δ4 transformed an h90mei2.16 strain to Leu+ at high frequency. The transformants displayed no observable morphological abnormality. This result implies that overexpression of mei3+ has little or no general cytotoxic effect.

The preceding experiment suggests, but does not demonstrate, that expression of mei3+ in a vegetative cell provokes haploid meiosis and sporulation in a wild-type but not a mei2.16 strain. Direct evidence that this is the case has been obtained. pmei3.15Δ4 was introduced into an h−5-leu1.32mei2.16 strain which was subsequently crossed with an h−5-leu1.32 strain. Spores derived from this cross were separated from unsporulated vegetative cells (see Materials and methods) and were germinated on minimal plates lacking leucine but supplemented with 50 mM 3′5′cAMP. This level of 3′5′cAMP has been shown previously to allow cells carrying a null-allele of ran1 to grow relatively normally (Beach et al., 1985) and therefore might be expected to rescue cells carrying pmei3.15Δ4 if the mei3+ gene product inactivates ran1+.

Only those Leu+ segregants which carried pmei3.15Δ4 through meiosis (~5%) were capable of forming colonies on minimal medium lacking leucine, supplemented with cAMP. Among these, ~90% had the mei2.16 marker but 10% did not. The mei2+, plasmid-bearing segregants, underwent dramatic haploid sporulation upon transfer to the same medium lacking 3′5′cAMP (Figure 7A). This behaviour precisely mimics that of a ran1ts mutant likewise removed from 3′5′cAMP medium at a non-permissive temperature (Beach et al., 1985; Figure 7A).

The effect of expressing mei3+ during spor germination was tested. A pure preparation of spores (Figure 7B), derived from the cross described above, was inoculated into minimal medium lacking both leucine and 3′5′cAMP. Most spores lacked the plasmid and therefore did not germinate and some, presumably those carrying the mei2.16 marker and the plasmid, grew and divided normally. However, a fraction of the spores germinated and became considerably swollen but did not undergo the apical outgrowth which usually precedes cell division. Instead they sporulated (Figure 7B). Again, this is precisely the behavior of spores, carrying a null or a temperature-sensitive allele of ran1, germinating in minimal medium at the non-permissive temperature (Beach et al., 1985; Figure 7B). These experiments demonstrate that the response of a vegetative cell to expression of the mei3+ gene is indistinguishable, by the criteria used, to its response to inactivation of ran1.

**Discussion**

Several conclusions can be drawn from the preceding experiments. (i) mei3+ is essential for meiotic initiation and pro-
bably for no other cellular function. (ii) The gene is expressed only in $h^+/h^-$ diploids, under nutritional conditions which allow expression of $matPi^+$. (iii) If mei3+ is expressed at high levels in actively dividing wild-type cells, growth is inhibited and haploid meiosis and sporulation follow. This phenotype is identical to that of recessive ranl mutants and is also suppressed by two conditions which suppress expression of the ranl phenotype: lack of mei2+ or addition of 3′5′cAMP to the growth medium.

The $matP^+$ and $matM^+$ alleles of the mating-type locus are not single genetic entities. Each contains two separate genes, known as $matPi^+\text{,} matPc^+\text{,} matMi^+\text{,} \text{and} matMc^+$ (i, inducible; c, constitutively expressed). A full description of the role of each of these genes in conjugation and sporulation, and also their nucleotide sequence, will be presented elsewhere. (Kelly, Smith, and Beach, in preparation). Here, we have demonstrated that $matPi^+$ (defined by the $matPi-102$ mutation (Bresch et al., 1968, Egel, 1984)), a gene that is essential for meiotic initiation, is required for mei3+ transcription (Figure 4C). Furthermore, the accumulation during meiosis of the mei3+ transcript and its translational product parallels the accumulation of $matPi^+$, which is expressed only in response to nitrogen starvation (Figures 5B and 6A). Transcriptional activation of the mei3+ gene is probably the major role of the mating-type genes in meiosis and sporulation, because expression of this gene alone is sufficient to initiate the process.

Expression of mei3+ in a vegetative cell, albeit at levels higher than during normal methods, bypasses both nutritional and mating-type requirements for meiosis and sporulation (Figure 7). Since inactivation of ranl is the only other condition which is known to cause haploid meiosis in fission yeast, we propose that mei3+ initiates meiosis by inhibiting ranl+. mei3 mutants are presumed to be meiotically defective because, in the absence of mei3+, ranl+ activity cannot be inhibited to the point required for meiotic initiation.

We have previously determined the nucleotide sequence of the ranl+ gene and find that it encodes a protein which shares sequence homology with known protein kinases (McLeod and Beach, 1987). This finding may provide a clue to the observed suppression of sporulation in both ranl1ts and mei3+ overexpressing strains exposed to high levels of 3′5′cAMP (Figure 7A; Beach et al., 1985). It has been proposed that ranl+ encodes a protein kinase which is distinct from 3′5′cAMP-dependent protein kinase, but which shares a subset of key substrates. These are expected to be phosphorylated during vegetative growth and dephosphorylated during meiotic initiation (Beach et al., 1985). Inactivation of ranl+ is presumed to allow dephosphorylation of the substrates, unless 3′5′cAMP dependent protein kinase is unusually active, in which case they remain phosphorylated and meiotic initiation is inhibited. The mechanism by which the 21-kd mei3+ gene product regulates the activity of the postulated ranl+ protein kinase, and the relationship between ranl+ and cAMP-dependent protein kinase will be the subject of future studies.

Developmental biologists have long recognized two stages during the differentiation of a specialized cell. These are generally known as cell or tissue determination which is followed, often after many cell divisions, by overt cellular differentiation. Our experiments begin to outline the molecular steps which interconnect these two processes in the meiotic pathway of fission yeast. The mating-type locus controls cell type determination ($h^+$, $h^-$ or $h^+/h^-$) through the regulation of expression of unlinked genes. The product of the ranl+ gene, which is probably a protein kinase, directly regulates meiotic differentiation. The mei3+ gene acts as the link which interconnects these two levels of control during the transition from vegetative growth to meiosis and sporulation.

Materials and methods
Strains and growth media
All strains of $S. pombe$ used in this study were derived from the original isolates $h^+$ (Kelly et al., 1974), $h^-$ (Kelly et al., 1977) and $h^+/h^-$ (Kelly et al., 1975) introduced by U. Leupold. Strains carrying the mei3.71 and ran1.114 alleles were derived from those provided initially by R. Egel and M. Yamamoto. The strains used in this study were as follows:

- $h^+$,leu1.32mei3.71ade6.210 (SP94),
- $h^+$,leu1.32ade6.210 (SP67),
- $h^+$,leu1.32ade6.210mei16.16 (SP149),
- $h^+$,leu1.32ade6.210met2.16 (SP772),
- $h^+$,leu1.32ade6.216 (SP202),
- $h^+$,ade6.210h+.6.216 (SP799),
- $h^+$,ade6.210h+.6.216 (SP797),
- h^+ranl.114 (SP399),
- h^+ranl.114 (SP402)

Yeasts were cultured either in rich medium (YEA; 0.5% yeast extract, 3% glucose, 75 μg/ml Adenine) or minimal medium (Mischion, 1970) buffered with 50 mM Sodium phosphate pH 5.6. The nitrogen or glucose level of the medium was adjusted as described in the text.

Genetic crosses and mating-type determinations were done as described in Gutz et al. (1974).

Nucleotide sequencing
All nucleotide sequence was obtained by means of the dideoxynucleotide method (Sanger et al., 1977) using [35S]dATP (New England Nuclear) and universal primer.

Single-stranded DNA template was prepared from the vector pUC118 carrying inserts of mei3+ DNA. pUC118 was derived from pUC18 (Yanish-Perron et al., 1985) by insertion of a 300-bp fragment of the intergenic region of $M\text{I3}$ into the unique Ndel site. The plasmid was obtained in single-stranded form following superflocculation of plasmid-bearing E. coli (TGI) with the phage M13KO7 (Vieira, personal communication).

In order to obtain a series of overlapping clones for sequencing, the EcoRV fragment carrying mei3+ was inserted in both orientations into the unique Smal site of pUC118. Unidirectional deletions of the inserted DNA were constructed according to the method of Henikoff (1984). Fully overlapping sequence was obtained from both strands.

Construction of the expression vector, pART1
pART1 is a fission yeast expression vector derived from pUC118. A 1.2-kb S. pombe $\text{ars}$ fragment (Losson and Lacroute, 1983) was inserted into the unique EcoRI site of the polylinker of pUC118. The LEU2 gene of S. cerevisiae (Beggs, 1978) was then inserted as a 2.2 kb fragment into the unique HindIII site, and a 700bp Sph/Pst restriction fragment carrying the promoter of the adh gene of $S. pombe$ (Russel, 1983) was inserted into the unique Sphi and Pstl sites of the polylinker. In part1, transcription was driven away from the Sphi site towards any sequence inserted into the remaining unique Psti, Safl, BanHI, Smal or Sfrl sites of the polylinker. The 5′ end of the pART1 transcript contained no AUG initiation codon, which therefore had to be provided by the inserted sequence. The end-points of deletions constructed in pART1 could be directly determined by dideoxynucleotide sequencing (Sanger et al., 1977) since pART1 could be isolated in single-stranded form. The oligonucleotide, 5′TTTGACCTCCCTCATGG, was synthesized as a sequencing primer for pART1. It anneals to the adh promoter region and is therefore well placed to sequence neighboring deletion end-points.

RNA isolation
Cells were harvested at an appropriate stage of growth (see text) and washed in ice-cold water. Ten ml sterile glass beads (0.45 μm diameter) were added to the pelleted cells and cold breaking buffer (0.32 M sucrose, 20 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5 mg/ml heparin) was added until the meniscus just covered the glass beads. The cells were broken by vortexing for 1 min. Twenty ml dilution buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% SDS, 0.5 mg/ml heparin) was added to the broken cells along with an equal volume of phenol. The mixture was vortexed vigorously and the aqueous phase isolated. The aqueous phase was extracted twice more with phenol and finally with phenol/chloroform. The RNA was precipitated from the aqueous solution following addition of LiCl to 0.5 M and ethanol to 70%. This method is derived from that of Beggs et al. (1980).

S1 mapping and Northern blotting
32P-labelled single-stranded DNA probes were prepared by primer extension according to the method of Burke (1984). The mei3+ DNA fragments were carried in pUC118 or pUC119. The precise limits of each probe are given in the legend to Figure 4.
Twenty μg total cell RNA was hybridized with [32P]DNA in 0.25 M NaCl, 0.3 M Hepes pH 7.6, 3 mM EDTA. Reactions were incubated for 16 h at 65°C.

The reaction mixture (100 μl) was electrophoresed on 4 mM ZnSO4, 30 mM NaCl, pH 4.6, 0.25 M NaCl and the protected fragments were resolved on 6% acrylamide/urea gels.

Northern blots were performed as described in Maniatis et al. (1982).

**Photomicroscopy**

For visualization of cell nuclei and spores cells were fixed in 70% ethanol for 18 h, rinsed in 50 mM sodium citrate pH 7.0, and incubated in 50 μg/ml RNase at 37°C for 1 h. Propidium iodide (Sigma) was added to 2μg/ml and the cells were rinsed and visualized phase and fluorescence microscopy. This procedure highlights cell nuclei. Fully mature spores fluoresce with exaggerated intensity because RNase fails to penetrate and degrade the RNA within these structures.

**Expression of mei3** in E. coli

The plasmid pAR3038 is a derivative pBR322 carrying 23 bp of the promoter of gene 10 of the bacteriophage T7. The T7 promoter is flanked by BglII and EcoRV restriction sites and is adjacent to a unique Ndel site that is suitable for the insertion of target genes (F.W. Studier, personal communication). The plasmid pHK172 was derived from pAR3038 by digestion with EcoRI site (4361 in pBR322) and EcoRV followed by flush ending with the Klenow fragment of DNA polymerase and blunt-end ligation. The resulting plasmid, which has a deletion extending from position 2066 in pBR322 and BglII and blunt-end ligated in order to remove the control region of pBR322. The unique BglII restriction site was found upstream of the mei3 gene. The mei3 gene was cloned as a Ndel/EcoRI fragment into pRK172 to give pME13.18.

The E. coli strain BL21(DE3) was used for expression of mei3 protein. The strain carries a chromosomal copy of the bacteriophage T7 RNA polymerase gene under the control of the lac/UV5 promoter (Studier and Moffatt, 1986).

Alkaline lysis was performed on E. coli strain BL21(DE3) carrying the mei3 gene fused to promotor (Studier and Moffatt, 1986) from gel slices isolated on 15% SDS polyacrylamide gels stained with Coomassie blue. Protein samples were reduced with 2-ME and resolved by electrophoresis on 12% SDS-PAGE gels. Western blotting was performed as previously described (Maniatis et al., 1982).