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

Key words: Schizosaccharomyces pombe/meiosis/*mei3*

Introduction

The fission yeast, *Schizosaccharomyces pombe*, has two primary cell types known as h^+ and h^- (Leupold, 1950). They differ only in the allele expressed at the mating-type locus, *matP*$^+$ in an h^+, and *matM*$^+$ in an h^- cell (Leupold, 1958). In wild-type strains (h^{90}; homothallic, 90% self-mating) *matP*$^+$ and *matM*$^+$ 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^+ and h^- cells fuse with each other and thereby form a third cell type, the h^+/h^- diploid zygote. Under certain conditions the h^+/h^- 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^+, h^- or h^+/h^-) is determined by the *matP*$^+$ and *matM*$^+$ genes of the mating-type locus, expression of the sexually differentiated state appears to be controlled by the *ran1*$^+$ gene (referred to as *pat1*$^+$ 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_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*$^+$ 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*$^+$/*matM*$^+$) and nutritional starvation, are rendered inessential following total loss of *ran1*$^+$. Since the events which occur in haploid cells following inactivation of *ran1*$^+$ bear many similarities to true meiosis (Iino and Yamamoto; 1985b, Beach *et al.*, 1985) it has been proposed that inactivation of *ran1*$^+$, under the control *matP*$^+$ and *matM*$^+$, is the key step which triggers meiotic initiation in h^+/h^- 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*$^+$ might be transcriptionally regulated by *matP*$^+$ and *matM*$^+$. However, the *ran1*$^+$ transcript was found to be equally abundant during vegetative growth and meiosis (McLeod and Beach, 1987). Thus, if inhibition of *ran1*$^+$ is indeed an essential step in meiotic initiation, the mating-type genes must influence *ran1*$^+$ indirectly, presumably by controlling the expression of one or more intermediate genes that which in turn regulate the activity of the *ran1*$^+$ gene product. *mei3*$^+$ is a candidate for this role.

In addition to *matP*$^+$ and *matM*$^+$, *mei2*$^+$ and *mei3*$^+$ 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*$^+$. 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*$^+$ does not interfere with expression of the *ran1ts* phenotype, whereas loss of *mei2*$^+$ suppresses both the growth defect and meiotic initiation in the *ran1ts* mutant. These observations suggest that *mei3*$^+$ acts 'upstream' and *mei2*$^+$ 'downstream' of *ran1*$^+$ in the meiotic pathway.

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

Results

Null-allele of mei3

The *mei3*$^+$ gene was isolated by transformation of a sporulation defective homothallic strain, h^{90}*leu1.32mei3.71* (Spo$^-$, Leu$^-$),

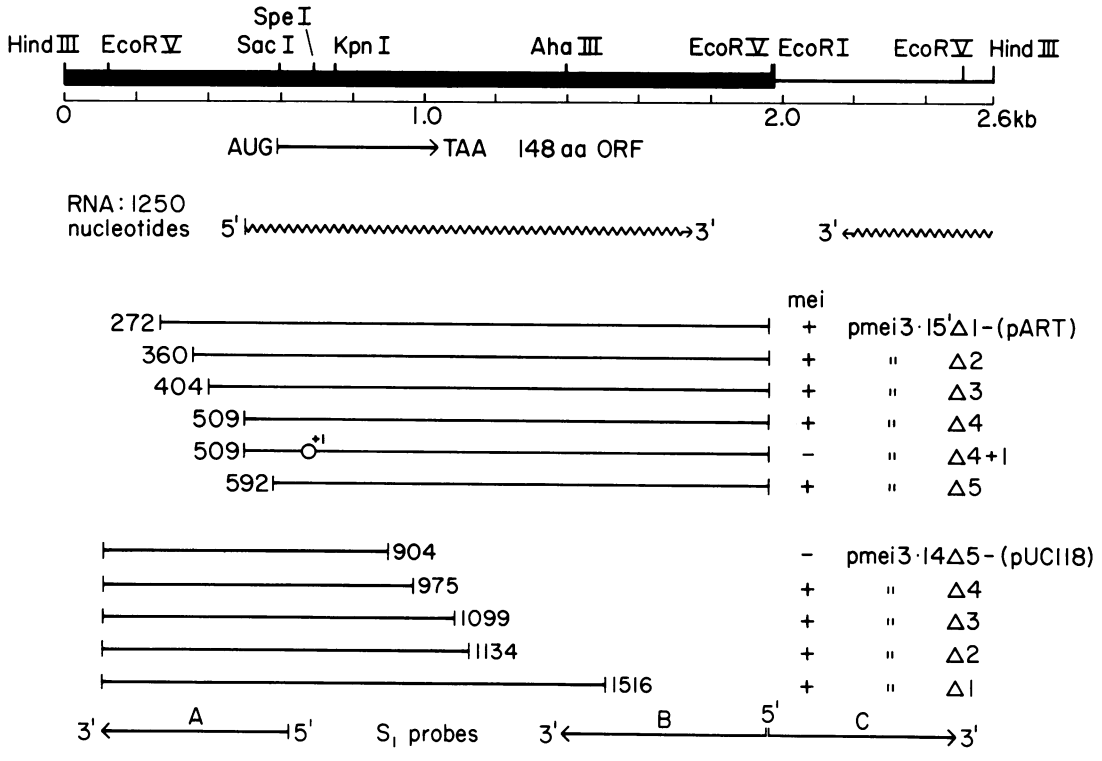

Fig. 1. Restriction map of a 2.6-kb *Hin*dIII restriction fragment containing the *mei3*$^+$ gene. The heavy line indicates the 1952-bp region which was sequenced. An ORF of 148 amino acid residues is marked. The 5′ and 3′ ends of the transcript of *mei3* and an adjacent gene are marked. The end points of deletions at the 5′ and 3′ end 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 pART.1 (*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 *Spe*I site in *mei3*. A, B and C indicate three single-stranded probes used for S_1 transcript mapping (Figure 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 *Hin*dIII 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 *Kpn*I site within this *Hin*dIII restriction fragment resulted in loss of Spo$^+$ activity (Figure 1). The *LEU2* gene of *S.cerevisiae* was inserted into this *Kpn*I site. This construction (*mei3*::*leu*) was returned to the chromosome of an *h*^{90}*leu1.32* 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, *h*^{90}leu1.32mei3::*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).

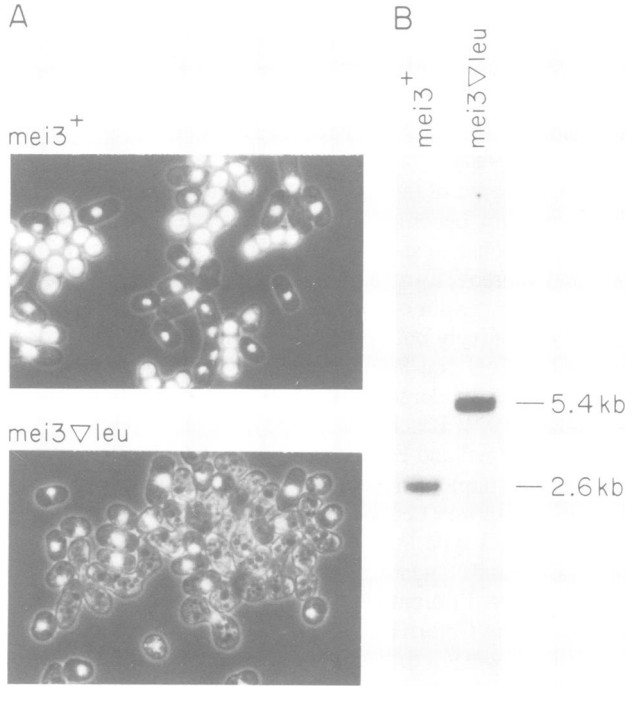

Fig. 2. Gene disruption of *mei3*. **(A)** Upper panel: zygote formation followed by meiosis and ascospore formation in a self-mating *h*^{90}*leu1.32* strain. **Lower panel:** *h*^{90}*leu-32mei3::leu* gene distruptant. This strain conjugates successfully, but forms abortive zygotes which fail to complete meiosis or sporulation. **(B)** Southern blot of *Hin*dIII-digested chromosomal DNA isolated from the same two strains shown in (A). The 2.6-kb *Hin*dIII *mei3* restriction fragment (Figure 1) was nick-translated and used as a hybridization probe. Insertion of a 2.8-kb *Bgl*II fragment containing the *leu2* gene into *mei3* increased the mol. wt of the hybridizing band from 2.6 to 5.4 kb.

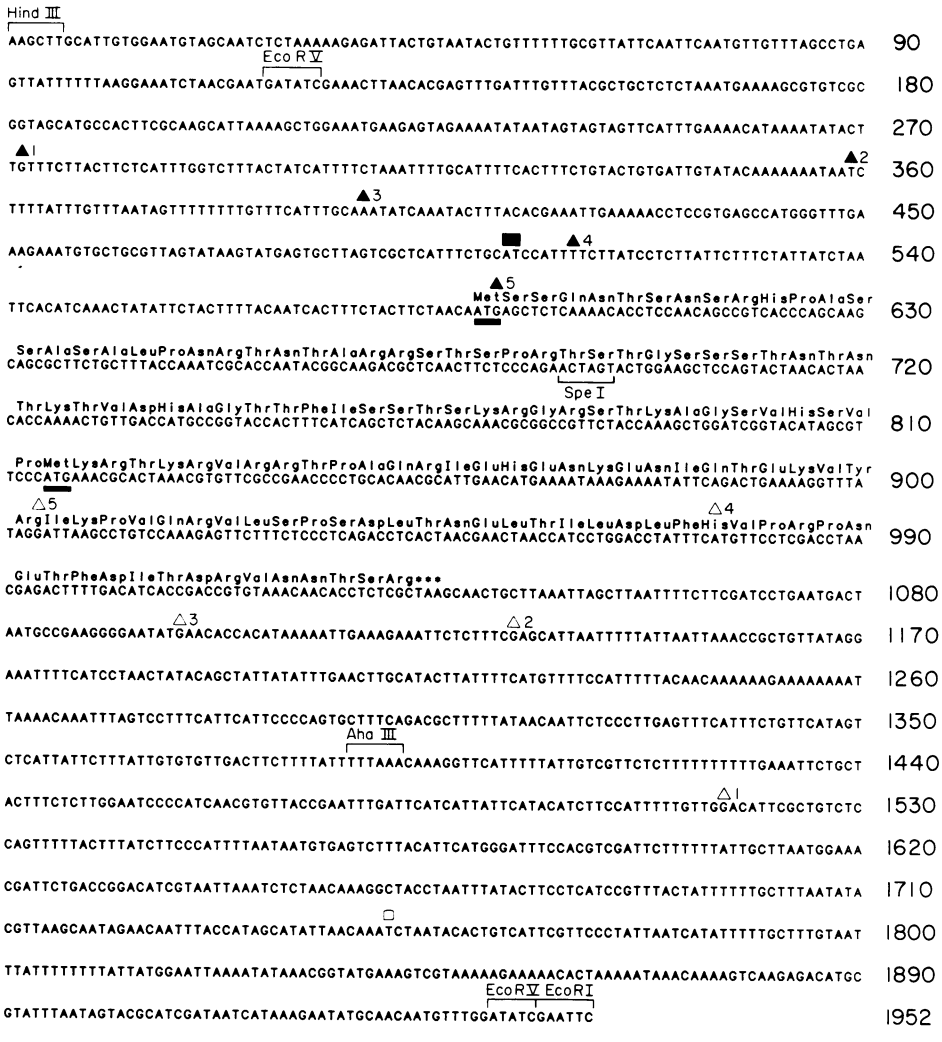

Fig. 3. Nucleotide sequence of a 1952-bp *HindIII/Eco*RI restriction fragment containing the *mei3*$^+$ gene. The predicted translational product of 148 amino acid residues is indicated. The initiating ATG and the ATG of Met 76 are underlined. The unique *Spe*I site is marked. The sites of transcriptional 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.

Structure of mei3

The nucleotide sequence of a 1952-bp *Hin*dIII/*Eco*RI subclone of the original 2.6-kb *Hin*dIII restriction fragment (Figure 1) was determined (Figure 3). The sequence contained no region of open-reading-frame (ORF) >444 bp, but this ORF contained the essential *Kpn*I site (Figure 1). RNA transcripts spanning this region of DNA were mapped, and an appropriate *Eco*RV restriction fragment (Figure 1) was subjected to detailed deletion analysis in order to establish whether the ORF might encode the *mei3*$^+$ gene product.

The 2.6-kb *Hin*dIII fragment hybridized with two transcripts, which were difficult to resolve on a Northern blot (Thomas, 1980) due to the similarity of their mol. wts (Figure 4E). S_1 mapping (Berk and Sharp, 1978) revealed that the two transcripts were convergent and not overlapping, but whereas one transcript of 1250 nucleotides lay entirely within the 2.6-kb *Hin*dIII fragment, the other was truncated at its 5′ end (Figures 1 and 4). Since subclones that contained no part of the truncated transcript retained full *mei3*$^+$ activity (Figure 1) we conclude that the centrally positioned 1250 nucleotide RNA is the transcriptional product of the *mei3*$^+$ gene. This transcript spans the essential *Kpn*I site and contains the 148 amino acid ORF.

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 pUC118 which is derived from pUC18 (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 *h*^{90}*leu1.32mei3.71* 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

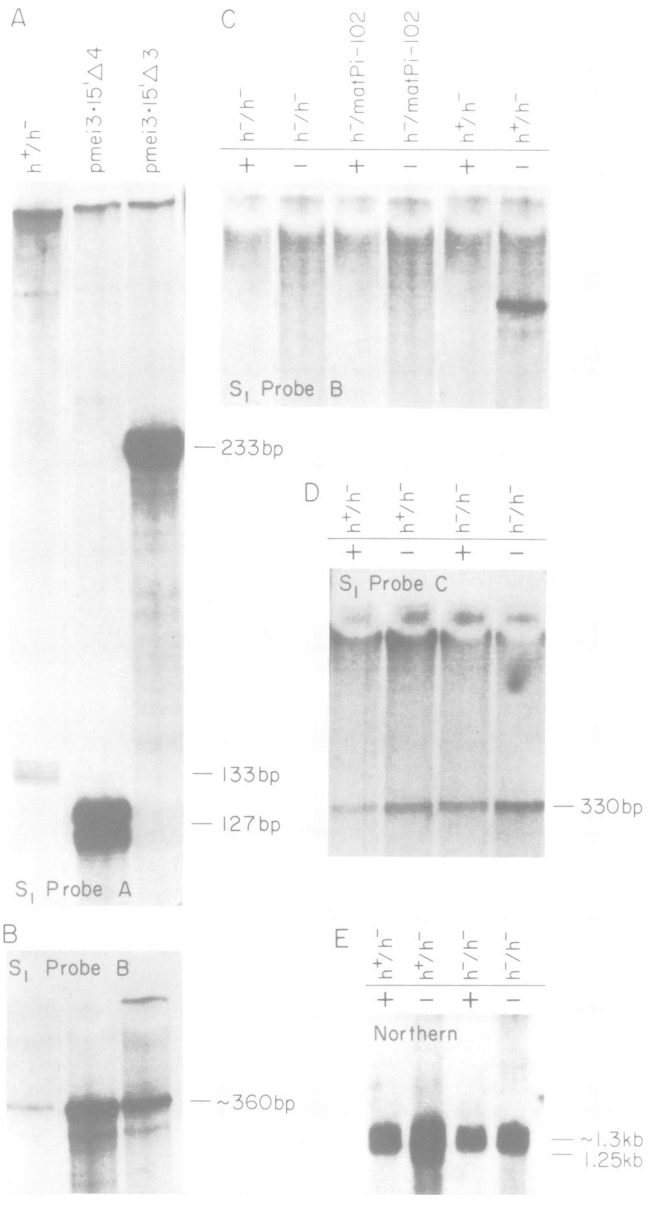

Fig. 4. Transcripts of *mei3* and an adjacent gene. (**A**) Probe A (Figure 1) protected from S_1 nuclease after annealing with RNA prepared from: **lane 1**, a strain entering meiosis (H^+/h^-); **lanes 2** and **3**, a vegetative *mei2.16* strain, expressing *mei3* under control of the *adh* promoter (*pmei3.15′Δ4*, *pmei3.15′Δ3*, Figure 1). The probe (A) was prepared by extension of an oligonucleotide primer which spanned nucleotides 634−608. The same primer was used to generate a DNA sequencing ladder which was used to determine the precise end-points of S_1 protection. This data established the position of the 5′ end of each transcript (Figures 1 and 3). (**B**) Probe B (Figure 1) protected from S_1 nuclease after being annealed with the same three RNA preparations described above. Probe B extends from an external *Eco*RV site to the *Aha*III site within *mei3* (Figures 1 and 3) and determines the position of the 3′ termination site of the transcripts. (**C**) Probe B (Figure 1) protected from S_1 nuclease after annealing with RNA prepared from: **lane 1**, h^{-S}*6.210*/h^{-S}*ade6.216* during vegetative growth (+); **lane 2**, same strain 6 h after transfer to nitrogen (NH_4Cl)-free medium (−); **lane 3**, h^{-S}*ade6.210*/h^{+N}*matPi-102* during vegetative growth (+); **lane 4**, same strain 6 h after transfer to nitrogen free medium (−). (**D**) Probe C (Figure 1) protected from S_1 nuclease after being annealed with RNA prepared from h^+/h^- or h^-/h^- strains cultured with a nitrogen source (+) or after 6 h of nitrogen starvation (−). (**E**) Northern blot of the same four RNA preparations described above, probed with a nick-translation of the entire 2.6-kb *Hin*dIII fragment (Figure 1). A transcript of ~1.3 kb is present in all four preparations, but the 1.25-kb transcript, which is poorly resolved from the 1.3-kb transcript, is present only in the h^+/h^- strain under nitrogen starvation (−).

criteria, deletions which removed the entire presumed 3′ untranslated region and up to 20 amino acids of the carboxy terminus of the 148 amino acid residue ORF (*pmei3.14Δ1-Δ4*) retained full *mei3* activity (Figures 1 and 3). However, a deletion which removed 43 amino acids of the ORF (*pmei3.14Δ5*) allowed sporulation in <0.1% of the Leu^+ transformant colonies (Figures 1 and 3). We conclude that deletions which remove no more than 20 residues of the ORF can rescue *mei3.71* in *trans* and therefore encode a biologically active polypeptide.

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′Δ1−Δ5*) retained $mei3^+$ activity (Figures 1 and 3). However, a +1 frameshift mutation, (*pmei3.15′ Δ4+1*) created by opening and filling in the 4-bp 5′ overhang of a unique *Spe*I 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 *Spe*I site, this codon must be used for translational initiation. It follows that the construction (*pmei3.15′Δ5*) which lacks this initiating codon must make use of the only other in-frame AUG at Met 76 and synthesize 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 S_1 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 (NH_4Cl) 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* (*matPi-102* see Discussion; Egel, 1984; Bresch *et al.*, 1968) did not transcribe the gene (Figure 4C).

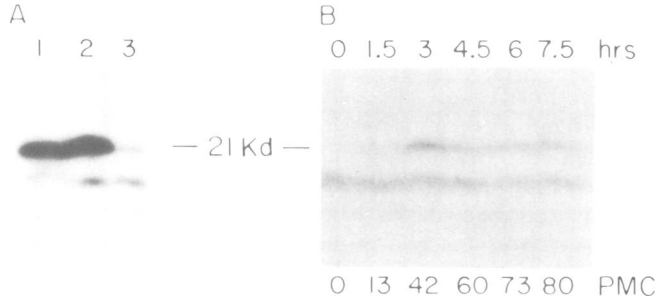

Fig. 5. Western blot of yeast protein using the N8 anti-*mei3* monoclonal antibody. **(A) Track 1:** *mei3*$^+$ protein purified from *E. coli*; **track 2**, whole cell lysate of yeast strain (SP149) carrying *pmei3.15'Δ4*; **track 3**, whole cell lysate of h^+/h^- strain (SP272) during meiosis. The 21-kd band represents the product of the *mei3*$^+$ gene. The lower band, present only in yeast lysate, indicates the existence of a protein which cross-reacts with the N8 antibody. The level of this protein is not responsive to changes in the level of the *mei3*$^+$ gene product. **(B)** Induction of 21-kd *mei3*$^+$ protein during meiosis. The time is given following the initiation of meiosis by addition of 0.1% glucose, 1% glycerol to the culture medium of the h^+/h^- strain (see legend to Figure 7 for protocol). The percentage of cells in the culture committed to meiosis percent meiotic commitment, PMC) at each time point is also indicated (see legend to Figure 7 for protocol).

A strain which has the potential to sporulate (h^+/h^-) also failed to express *mei3*$^+$ both during vegetative growth and after entering asexual 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 non-meiotic 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 *matPi*$^+$ is essential for *mei3*$^+$ transcription (Figure 4C) it was of interest to compare the accumulation of the *mei3*$^+$ and *matPi*$^+$ 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 azygotic asci). During this period the *matPi*$^+$ and *mei3*$^+$ transcripts accumulated synchronously but whereas the level of the *mei3*$^+$ transcript was stable after 2.5 h, the *matPi*$^+$ 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 *Hin*dIII 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).

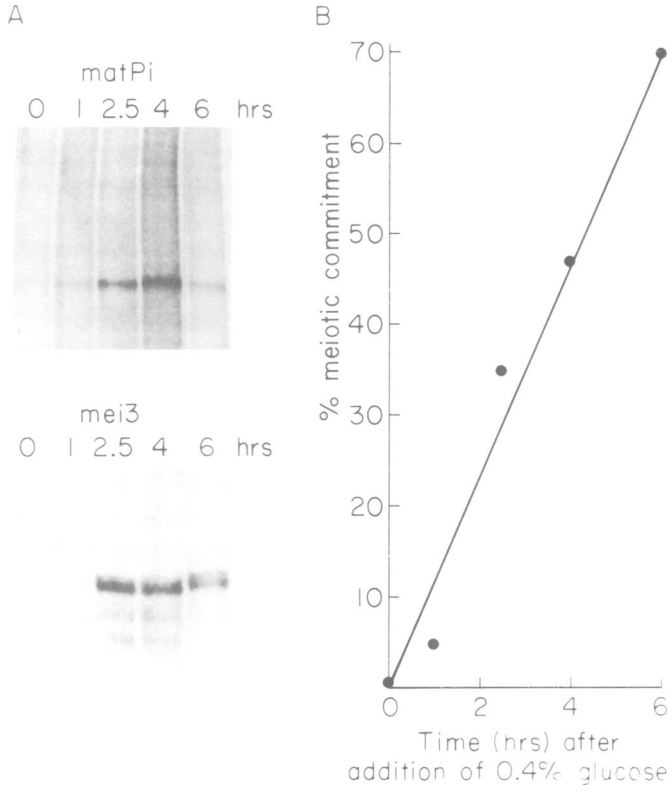

Fig. 6. Induction of the *mei3*$^+$ and *matPi*$^+$ transcripts during meiosis. **(A)** Transcripts of *matPi*$^+$ (upper panel) and *mei3*$^+$ (lower panel) in an h^{+N}*ade6.210*/h^{-S}*ade6.216* strain (SP272) entering meiosis. The yeast was cultured to stationary phase in minimal medium (Mitchison, 1970) containing 3% glucose and 100 mM NH_4Cl. Meiosis is not initiated in the medium even at stationary phase. Stationary phase cells were washed and incubated in minimal medium lacking both carbon (glucose) and nitrogen (NH_4Cl) sources. Meiosis was initiated by addition of glucose to 0.1% and glycerol to 1%. RNA was annealed with probe A (*mei3*) or a region of the *matPi* gene. **(B)** Meiotic commitment in the same culture from which RNA was isolated. Commitment was assayed as follows. The h^{+N}*ade6.210*/h^{-S}*ade6.216* diploid is phenotypically Ade$^+$, due to intragenic complementation of the *210* and *216* alleles (Gutz *et al.*, 1974). At intervals after glucose/glycerol addition, cells were plated on complete minimal medium containng only 10 μg/ml adenine. This medium supports growth of Ade$^-$ colonies but they develop a pink colour. Diploid cells which were committed to the completion of meiosis and sporulation at the time of plating segregate *ade6.210* and *ade6.216* spores which germinate and form mixed colonies. Both cell types are Ade$^-$ and turn pink. Diploids not committed to meiosis at the time of plating form colonies which contain only *ade6.210/ade6.216* diploid cells and remain white. The ratio of pink:total colonies, at each time interval after glucose addition, gives an estimate of meiotic commitment.

Vegetative expression of mei3$^+$ *phenocopies ran1 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 h^{90}) and a *mei3.71* strain of yeast to Leu$^+$, at a frequency 100- to 1000-fold lower than the same vector lacking the *mei3* insert.

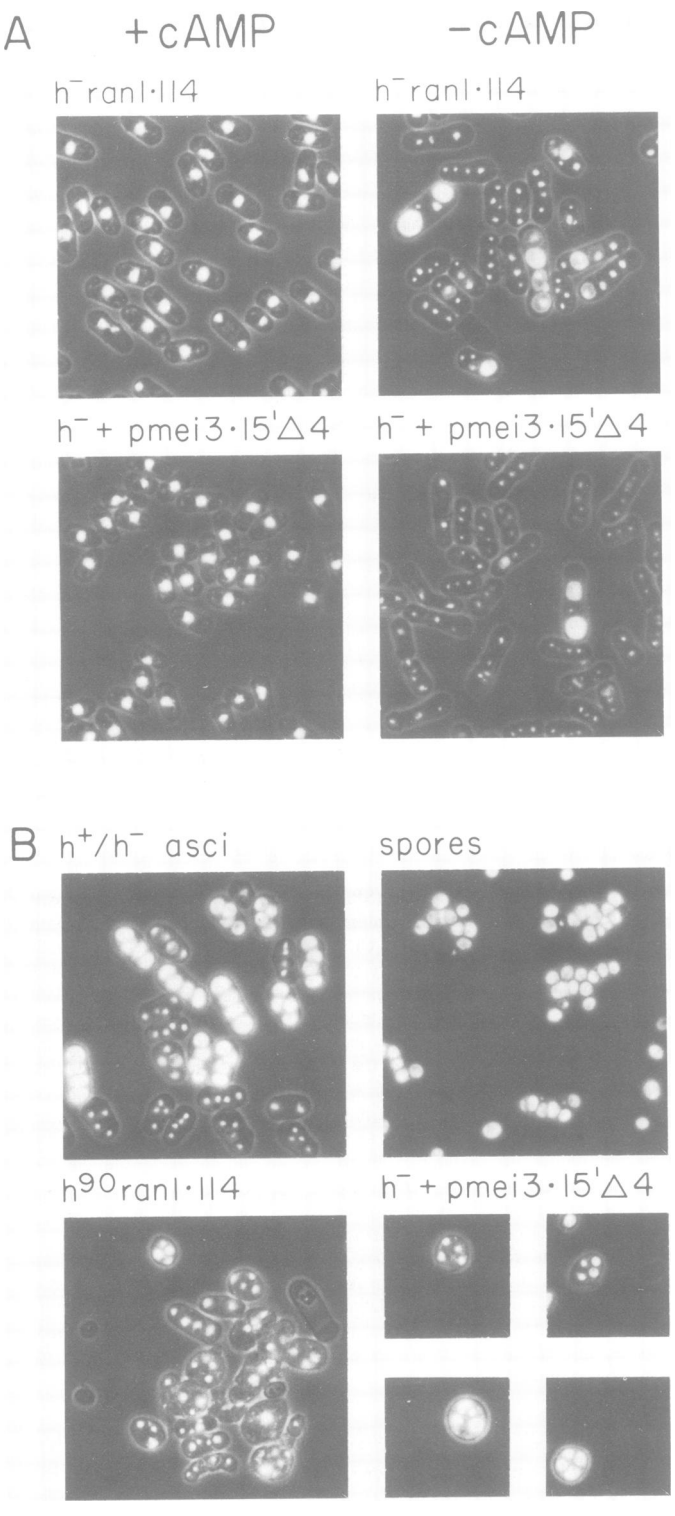

Fig. 7. Comparison of the phenotype of a *ran1ts* mutant and a wild-type strain in which the *mei3*$^+$ gene is overexpressed. **(A) 1**, h^{-S}*ran1.114* haploid strain cultured at a non-permissive temperature (33°C) in minimal medium supplemented with 50 mM 3′5′cAMP. **2**, Same culture 10 h after removal of cAMP. **3**, h^{-S}*leu1.32* haploid strain carrying *pmei3.15′Δ4*, cultured with cAMP. This strain is a segregrant from the cross described in the text. **4**, Same strain 10 h after removal of cAMP. **(B) 1**, h^{+N}*ade6.210*/h^{-S}*ade6.216* diploid strain entering meiosis. The 'azygotic' asci differ from those in Figure 2A because the zygote was propagated vegetatively and sporulation was not immediately preceded by conjugation. **2**, Preparation of spores purified according to the method of Beach *et al.* (1985). **3**, Haploid spores of an h^{90}*ran1.114* strain germinating and subsequently sporulating in minimal medium at 34°C. **4**, Four individual germinating spores which are presumed to be carrying *pmei3.15′Δ4* (see text).

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 (*pmei3.15′Δ4+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, *pmei3.15′Δ4* transformed an h^{90}*mei2.16 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. *pmei3.15′Δ4* was introduced into an h^{-S}*leu1.32mei2.16* strain which was subsequently crossed with an h^{+N}*leu1.32* strain. Spores derived from this cross were separated from unmated 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 *pmei3.15′Δ4* if the *mei3*$^+$ gene product inactivates *ran1*$^+$.

Only those Leu$^+$ segregants which carried *pmei3.15′Δ4* 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 spore 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 *ran1* mutants and is also suppressed by two conditions which suppress expression of the *ran1* 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*$^+$, *matPc*$^+$, *matMi*$^+$ 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 *ran1* is the only other condition which is known to cause haploid meiosis in fission yeast, we propose that *mei3*$^+$ initiates meiosis by inhibiting *ran1*$^+$. *mei3* mutants are presumed to be meiotically defective because, in the absence of *mei3*$^+$, *ran1*$^+$ activity cannot be inhibited to the point required for meiotic initiation.

We have previously determined the nucleotide sequence of the *ran1*$^+$ 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 *ran1ts* and *mei3*$^+$ overexpressing strains exposed to high levels of 3′5′cAMP (Figure 7A; Beach *et al.*, 1985). It has been proposed that *ran1*$^+$ 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 *ran1*$^+$ 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 *ran1*$^+$ protein kinase, and the relationship between *ran1*$^+$ 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 *ran1*$^+$ 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^{90} (968), h^{-S} (972) and h^{+N} (975) 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^{90}*leu1.32mei3.71ade6.210* (SP94),
h^{90}*leu1.32ade6.210* (SP67),
h^{90}*leu1.32ade6.210mei2.16* (SP149),
h^{-S}*leu1.32ade6.210mei2.16* (SP572),
h^{+N}*leu1.32ade6.216* (SP202),
h^{-S}*ade6.210/h*$^{-S}$*6.216* (SP299),
h^{-S}*ade6.210/h*$^{+N}$*ade6.216* (SP272),
h^{+N}*matP.102ade6.216/h*$^{-S}$*ade6.210* (SP585),
h^{-S}*ran1.114* (SP399),
h^{90}*ran1.114* (SP402)

Yeasts were cultured either in rich medium (YEA; 0.5% yeast extract, 3% glucose, 75 μg/μl Adenine) or minimal medium (Mitchison, 1970) buffered with 50 mM Sodium pthallate 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 [α ^{35}S]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 M13 into the unique *Nde*I site. The plasmid was obtained in single-stranded form following superinfection of plasmid-bearing *E. coli* (TGI) with the phage M13K07 (Vieira, personal communication).

In order to obtain a series of overlapping clones for sequencing, the *Eco*RV fragment carrying *mei3* was inserted in both orientations into the unique *Sma* 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 ars* fragment (Losson and Lacroute, 1983) was inserted into the unique *Eco*RI 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 *Hin*dIII site, and a 700-bp *Sph/Pst* restriction fragment carrying the promoter of the *adh* gene of *S. pombe* (Russell, 1983) was inserted into the unique *Sph*I and *Pst*I sites of the polylinker. In pART1, transcription was driven away from the *Sph*I site towards any sequence inserted into the remaining unique *Pst*I, *Sal*I, *Bam*HI, *Sma*I or *Sst*I sites of the polylinker. The 5′ end of the pART1 transcript contained no AUG initiation sequence, which therefore had to be provided by the inserted gene. 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′TTTGACGCCTCCCATGG, 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

^{32}P-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 [^{32}P]DNA in 0.25 M NaCl, 0.32 M Hepes pH 7.6, 3 mM EDTA. Reactions were incubated for 16 h at 65°C. The hybrids were digested with 100 U S_1 (BRL) in 4 mM $ZnSO_4$, 30 mM NaOAc 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 visualized under combined 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 *Bgl*II and *Eco*RV restriction sites and is adjacent to a unique *Nde*I site that is suitable for the insertion of target genes (F.W.Studier, personal communication). The plasmid *pRK172* was derived from *pAR3038* by digestion with *Eco*RI (position 4361 in *pBR322*) and *Eco*RV followed by flush ending with the Klenow fragment of DNA polymerase and blunt-end ligation. The resulting plasmid, which has a deletion in the promoter region of the tetracycline resistance gene was further digested with *Pvu*II (position 2066 in *pBR322*) and *Bgl*II and blunt-end ligated in order to remove the copy number control region of *pBR322*. The unique *Bam*HI site located downstream of the *T7* promoter region was filled in with Klenow polymerase and an *Eco*RI linker was added at this site to give *pRK172* (R.Kostriken, personal communication). *pRK172* therefore contains the *T7* gene 10 promoter but is propagated at very high copy number in *E. coli* due to removal of the pBR322 copy number control region.

An *Nde*I site was created at the initiating methionine of the *mei3*$^+$ gene by oligonucleotide mutagenesis of the plasmid *pmei3.14'*. This consists of pUC118 carrying the *mei3*$^+$ gene as an *Eco*RV restriction fragment (Figure 1). Single-stranded DNA was obtained from this plasmid and annealed with the 23 mer 5'TTTCTACTTCTACATATGAGCTC3'. Mutagenesis was as described (Zoller and Smith, 1984) and resulted in *pmei3.14'(Nde)* which is identical to *pmei3.14'* except that it contains an *Nde*I site (5'CATATG3') at the initiating methionine of the gene. *mei3*$^+$ was cloned as a *Nde*I/*Eco*RI 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). The plasmid *pME13.18* was introduced into this strain and ampicillin-resistant colonies were obtained. Individual transformants were grown at 37°C in 10 ml L Broth containing 50 μg/ml ampicillin. When the culture reached an OD_{660} of 0.2, glycerol was added to a final concentration of 15% and the cells were frozen at −70°C. For production of the *mei3*$^+$ protein, frozen cells were thawed and 10 λ innoculated into 10 ml M9 media (Miller, 1972) containing ampicillin. The culture was induced with 0.4 mM IPTG (Sigma) when attaining an OD_{660} of 0.4 and cells were harvested by centrifugation 3 h after induction. Cell pellets were stored frozen at −70°C.

Purification of mei3$^+$ protein

Frozen pellets (10-ml cells) were thawed on ice and resuspended in 1.0 ml 50 mM Tris−HCl pH 7.8, 2 mM DTT, 5 mM EDTA, 2 mM benzamidine HCl, 2 mM PMSF 10% glycerol, 0.3% Triton X100 and 0.6 mg/ml lysozyme (Sigma). After incubation on ice for 30 min, cells were sonicated for four 3-s intervals. The extract was clarified by centrifugation at 10 000 *g* for 20 min. Approximately 60% of the *mei3*$^+$ protein was found in the insoluble pellet and the material was solubilized in 1% sodium deoxycholate. The solubilized material consisted primarily of *mei3* protein and one other major contaminant. The contaminant was removed by DE52 cellulose (Whatman) chromatography.

Preparation of monoclonal antibodies

BalbC female mice (National Cancer Institute) were immunized with *mei3*$^+$ protein according to the following schedule. The first i.p. injection contained 50 μg *mei3*$^+$ protein in complete Freund's adjuvant. This was followed at 2-week intervals by further i.p. injections. Three weeks following the last injection, mice were immunized with 50 μg *mei3*$^+$ protein i.v. Mice were sacrificed 3 days after the final injection and spleen cells were fused in the presence of polyethylene glycol to NS-1 myeloma cells by standard techniques (Galfre *et al.*, 1977).

Hybridomas secreting antibodies specific for *mei3*$^+$ were identified by solid-phase radioimmunassay (Harlow *et al.*, 1985). Sixteen independent lines were identified in this manner.

Western blotting

Yeast cells were collected by centrifugation and frozen at −70°C. Cell pellets were thawed on ice and resuspended in 10 ml trichloroacetic acid (TCA). Glass beads (0.45 μm) were added to just cover the meniscus and the cells were broken by vortexing for 3 min. After 30 min incubation on ice, TCA precipitable material were collected by centrifugation. The pellets were washed extensively in H_2O-saturated ether and resolubilized in 5% SDS. Extract from 2 × 10^8 cells was diluted 1:1 in 2 × sample buffer (Laemmli, 1970). After boiling for 2 min, samples were electrophoresed on a 7.5−15.0% gradient polyacrylamide gel (Laemmli, 1970). Proteins were transferred to nitrocellulose (0.1 μm Schleicher and Schul) as described in Burnette (1981). Following transfer, the nitrocellulose sheets were blocked in 3% ovalbumin (Sigma) in phosphate-buffered saline (PBS) at 37°C for 2 h. Papers were washed in 150 mM NaCl, 5 mM EDTA, 0.25% gelatin 0.05 NP-40, 50 mM Tris 7.5 (NET) for 30 min at room temperature and then incubated with tissue culture supernatant containing anti *mei3*$^+$ monoclonal (N−8) for 1 h at room temperature. After washing three times in NET, the filter was incubated with 20 μCi ^{125}I rabbit anti-mouse IgG (New England Nuclear) in PBS, 1% BSA for 30 min at room temperature. After extensive washing in NET, filters were dried and exposed to photographic film.

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