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Interaction between *ran1*$^+$ protein kinase and cAMP dependent protein kinase as negative regulators of fission yeast meiosis

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In fission yeast, meiosis is initiated by transcriptional activation of the *mei3*$^+$ gene under the combined influence of the four mating type genes. The *mei3*$^+$ gene product acts as a meiotic inducer by binding to and inhibiting the *ran1*$^+$ protein kinase. Inactivation of *ran1*$^+$ kinase is both necessary and sufficient to allow meiotic differentiation. We describe a class of mutants which are unable to undergo both normal meiosis and meiosis induced by inactivation of *ran1*$^+$. In addition to these defects, the cells are sterile and unable to enter stationary phase. We have determined that the mutants define two complementation groups, designated *cgs1*$^+$ and *cgs2*$^+$ (continues to grow in stationary). The wild type allele of each gene has been isolated and sequence analysis of *cgs1*$^+$ shows that it encodes a protein homologous to the regulatory subunit of cyclic AMP dependent protein kinase (cAPK). Biochemical studies demonstrate that in *cgs1-1* containing cells, cAPK activity is unregulated by cyclic AMP (cAMP). Sequence analysis of *cgs2*$^+$ shows that the predicted protein it encodes shares homology with a phosphodiesterase from *Dictyostelium discoideum* and biochemical studies demonstrate that cells containing a mutant allele of *cgs2*$^+$ have elevated levels of cAMP. Thus, both genes encode proteins that regulate the activity of cAPK. We have previously shown that cells overproducing *ran1*$^+$ kinase are meiotically defective. Here, we provide direct evidence that the meiotic defect caused by either unregulated cAPK activity or unregulated *ran1*$^+$ kinase activity is due to inability to induce transcription of the *mei2*$^+$ gene, which is required for meiotic initiation. We propose that the switch from vegetative growth to meiosis in fission yeast requires inactivation of *ran1*$^+$ kinase and is prevented by unregulated levels of cAPK.

Key words: cAMP phosphodiesterase/kinase regulatory subunit/meiotic commitment/*ran1*$^+$ protein kinase/*Schizosaccharomyces pombe*

Introduction

Exit from the mitotic cycle and entry into meiosis in fission yeast is regulated by both nutritional signals and cell type. Haploid cells divide vegetatively as one of two mating types designated h^+ or h^- (Leupold, 1950). Cells of opposite mating type conjugate under conditions of nutrient deprivation to form a transient diploid zygote (Egel, 1971). The diploid zygote undergoes meiosis if starvation conditions continue and develops into an ascus containing four haploid spores.

The mating type of a cell is determined by the alleles expressed at the *mat1* locus, either *mat1-P*, specifying h^+ cell type or *mat1-M*, specifying h^- cell type (see Egel, 1989 for review). Each has been shown to contain two divergently transcribed genes referred to as P_i and P_c, and M_i and M_c. All four of the mating type genes are required for meiosis and sporulation (Kelly *et al.*, 1988). Each of the four mating type genes is transcriptionally regulated by nutritional signals and full activation of each is achieved only under conditions of nutrient limitation (Kelly *et al.*, 1988). Nutritional regulation of meiosis is more extensive than regulation of the mating type genes however, and at least one other gene required for meiosis, *mei2*$^+$, is transcriptionally induced during conditions of nutrient limitation (Shimoda *et al.*, 1987). In common with the mating type genes, *mei2*$^+$ has no known role during vegetative growth of the cells, but is required for premeiotic DNA synthesis. The biochemical role of the *mei2*$^+$ gene product during meiosis is currently unknown.

The mating type genes act, either directly or indirectly, as transcriptional activators of at least one and probably many unlinked genes. The *mat* locus regulates the *mei3*$^+$ gene which is a crucial meiotic inducer (McLeod *et al.*, 1987). The *mei3*$^+$ gene induces meiosis by regulating the activity of the *ran1*$^+$ protein kinase (McLeod and Beach, 1988; McLeod, 1989). The *ran1*$^+$ gene (also referred to as *pat1*$^+$) encodes a critical negative regulator of meiosis (Nurse, 1985; Iino and Yamamoto, 1985a). A model describing the function of *ran1*$^+$ and *mei3*$^+$ in regulation of meiosis is as follows. During vegetative growth, as nutrients become limiting, the signal of starvation is transmitted to the cell resulting in the partial inhibition of *ran1*$^+$ activity thus leading to conjugation. In the diploid zygote that results, *ran1*$^+$ activity is further inhibited by the product of the *mei3*$^+$ gene allowing meiosis to proceed. The product of the *ran1*$^+$ gene is a 52 kDa protein kinase capable of autophosphorylation *in vitro* (McLeod and Beach, 1988). Inhibition of *ran1*$^+$ kinase activity during meiosis is accomplished by a non-covalent association of the $p52^{ran1}$ kinase with the 21 kDa product of the *mei3*$^+$ gene (McLeod and Beach, 1988). Inhibition of $p52^{ran1}$ by $p21^{mei3}$ thus bears a mechanistic similarity to the regulation of cAMP dependent protein kinase by heat stable inhibitor (Beebe and Corbin, 1989). A formal similarity may be more extensive than this however, since gross stimulation of cyclic AMP dependent protein kinase (cAPK) by inclusion of large amounts of cAMP in the media prevents normal meiosis and bypasses the requirement for *ran1*$^+$ for vegetative growth (Beach *et al.*, 1985). A close interaction between the two pathways is expected. To examine the relationship between

ran1$^+$ kinase and cAPK, we are analyzing suppressors of *ran1-114* as a means of isolating further elements of the pathway.

Results

Physiology of strains unable to undergo meiosis

Previous observations had indicated that agents which stimulate cAPK in fission yeast inhibit normal meiosis (Calleja *et al.*, 1980; Beach *et al.*, 1985) and bypass the requirement of the *ran1*$^+$ gene product for vegetative growth (Beach *et al.*, 1985). In addition, fission yeast cells harboring plasmids encoding sequences for *Saccharomyces cerevisiae* cAPK become highly elongated during vegetative growth, especially as they approach stationary phase (Beach *et al.*, 1985) presumably due to an inability to respond normally to limiting nutrients by ceasing growth. To study the role of CAPK in meiosis, these observations were used as a rationale to obtain mutants that might be defective in genes encoding components of the cAPK pathway.

Cells containing extragenic suppressors that allow *ran1-114*-containing cells to grow at a fully restrictive temperature were selected (see Materials and methods). Cells were examined microscopically to identify any exhibiting the elongated shape characteristic of cells containing agents which stimulate cAPK. Three candidates were discovered in this manner, all of which were also sterile and meiotically defective (data not shown). Genetic analysis of each revealed that all were recessive to wild type with respect to both sterility and elongated cell morphology and that two complementation groups, designated *cgs1*$^+$ and *cgs2*$^+$ (continues to grow in stationary), were represented.

Wild type fission yeast cells arrest in stationary phase as small cells when nutrients in the media are expended. In contrast, *cgs* cells are elongated in stationary phase (Figure 1A) and cultures grown to high density accumulate many dead cells. Each mutant strain was further characterized to determine if, as these observations indicated, *cgs* cells were unable to enter stationary phase. Cells which have entered a G_0 stationary phase state are characterized by long term survivability (Baserga, 1976; Costello *et al.*, 1986). This criteria was used to compare the ability of cells containing either the *cgs1-1* or the *cgs2-1* allele to enter G_0 with that of wild type strains. Cells of both strains were cultured to stationary phase in four different media, which varied in either the amount of nitrogen source (NH_4Cl) or carbon source (glucose). At daily intervals thereafter, the viability of cells from each culture was determined by plating a portion of each onto fresh medium. The density at which cells reached stationary phase varied depending on the particular media used but was $\sim 1 \times 10^7$ cells/ml. The long term viability of wild type cells was excellent in any of the four media used; however, cells containing either *cgs1-1* or *cgs2-1* varied in their ability to survive periods of time in stationary phase depending on the composition of the medium at the time of entry into stationary (Figure 1, panel B).

The original screen used to identify *cgs* cells was based on the ability of the mutant strain to suppress the growth defect observed in a *ran1-114* strain plated at the restrictive temperature. It was therefore of interest to determine if *cgs* strains would also suppress meiosis and sporulation caused by loss of *ran1*$^+$. Double mutant strains of the composition *ran1*^{ts} *cgs1-1* and *ran1*^{ts} *cgs2-1* were constructed and each was assessed for the ability to undergo meiosis at the restrictive temperature for *ran1* (34°C). In this experiment, the cells were cultured in complete minimal medium at 25°C until a density of 1×10^6 was reached. The cells from both cultures were then divided into each of the four media described above and simultaneously shifted to 34°C. At 4 h intervals thereafter, the cells were examined microscopically to determine the number of cells containing spores.

The *ran1-114* strain entered meiosis within 4 h after the temperature shift and 80% of the cells eventually completed sporulation (Figure 2A). The *ran1 cgs1-1* and *ran1 cgs2-1* double mutant strains failed to undergo meiosis and sporulation and $<15\%$ of the cells examined contained spores (Figure 2B and C). Inhibition by *cgs1-1* or *cgs2-1* or a *ran1-114* induced meiosis is not nutritionally dependent although cells cultured under limiting nitrogen conditions undergo meiosis earlier and to a slightly higher extent than those grown in media not limited for nitrogen. Nitrogen starvation also caused the *ran1-114*-containing cells to undergo meiosis earlier than cells not limited for nitrogen, indicating that commitment to meiosis requires an additional event besides turning off *ran1*$^+$ and that this event is regulated by nitrogen starvation. We conclude that both *cgs1*$^+$ and *cgs2*$^+$ are involved in exit from the mitotic cycle and entry into either the G_0 stationary phase or the developmental pathway of meiosis.

Structure of cgs1$^+$

The *cgs1*$^+$ gene was isolated by transformation of the sporulation defective homothallic strain, SP491 (*h*^{90} *leu1-32 cgs1-1*; spo$^-$, Leu$^-$), to Leu$^+$ with a bank of wild type fission yeast DNA carried in the yeast/bacterial shuttle vector, pWH5 (Wright *et al.*, 1986). Approximately 1×10^4 transformants were examined after exposure to iodine vapors to identify spore-containing colonies. Nine Spo$^+$ transformants were obtained and plasmids from each were rescued into *Escherichia coli*. Only two of the plasmids recovered, pCGS1.1 and pCGSX.1, were able to rescue the *cgs1-1* sporulation defect after retransformation into yeast. We conclude that the remaining seven plasmids were yeast cotransformants and that the plasmids conferring a spo$^+$ phenotype in yeast were not recovered into *E.coli*. The two plasmids contained no extensive sequence homology, as determined by restriction enzyme analysis, and only pCGS1-1 contained sequences closely linked to the *cgs1*$^+$ gene (data not shown). A 2.5 kb *Hin*dIII fragment carried on pCGS1-1 was identified that was able to rescue the sporulation defect of a *cgs1-1* strain.

The nucleotide sequence of the *Hin*dIII fragment was determined (Figure 3). Translation of the sequence in all three reading frames revealed two large open reading frames capable of encoding peptides of 45 amino acids (Figure 3, nucleotides 410–544) and 373 amino acids (Figure 3, nucleotides 789–1907). To determine the precise structure of the gene, a cDNA copy of the *cgs1*$^+$ transcript was synthesized using an oligonucleotide primer (P4; see Figure 3 and Materials and methods) complimentary to the anticipated 3′ end of the gene. The cDNA synthesized was amplified in a polymerase chain reaction (Innis *et al.*, 1988) using a second primer (P1; see Figure 3 and Materials and methods) corresponding to the 5′ end of the amino-terminal open reading frame identified along with primer P4. The

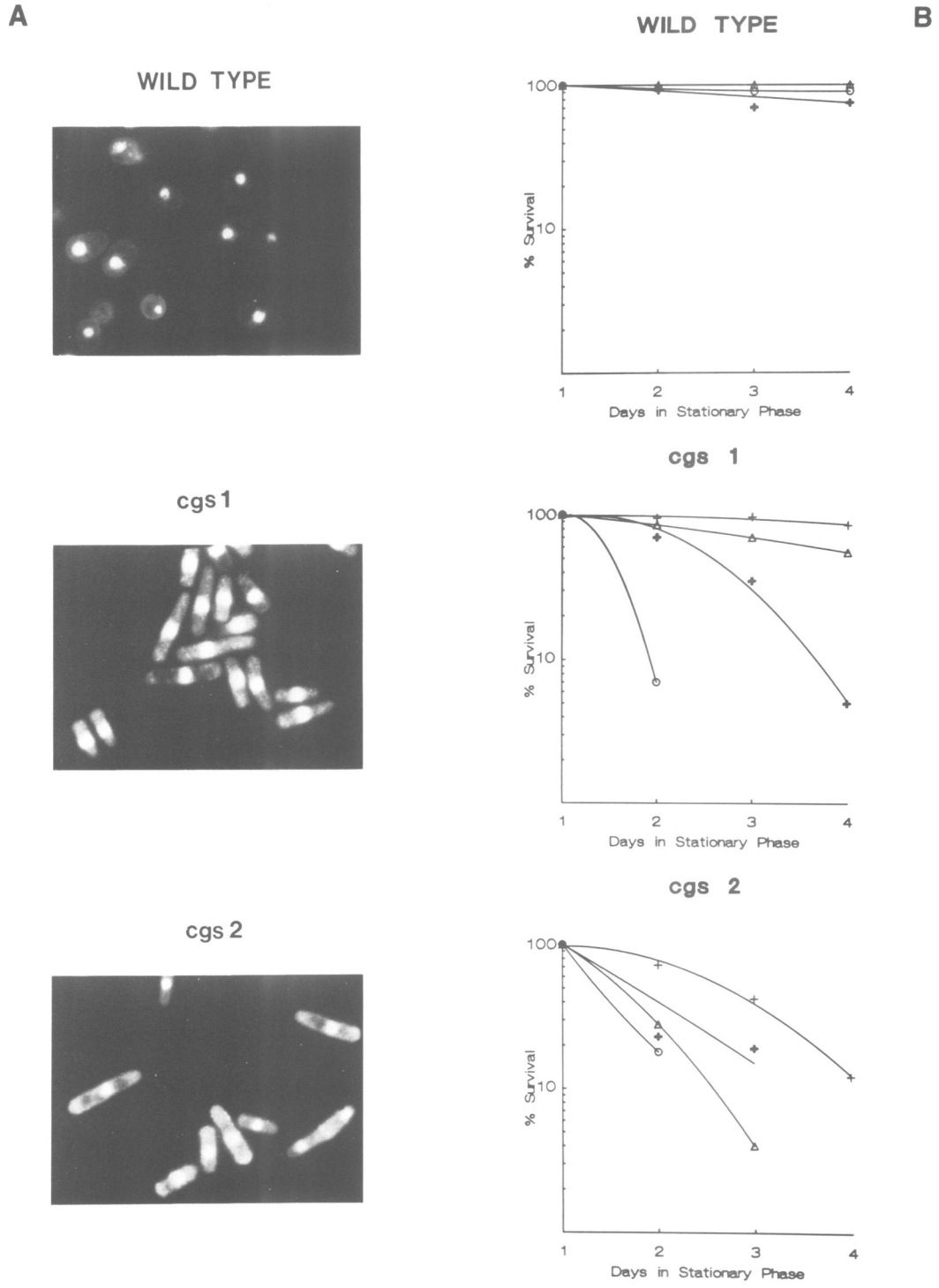

Fig. 1. *cgs* strains contain elongated cells and are unable to enter stationary phase. Panel **A**: Fluorescence micrographs of wild type (SP972), *cgs1-1* (SP512) and *cgs2-1* (SP489) cells stained with DAPI. Panel **B**: wild type (SP972), *cgs1-1* (SP512) and *cgs2-1* (SP489)-containing cells were grown at 30°C in either (▽)PM [2% glucose, 100 mM NH_4Cl]; (○) high glucose media [4% glucose, 100 mM NH_4Cl]; (+) low glucose media [0.2% glucose, 100 mM NH_4Cl] or (+) media containing 20-fold less nitrogen [2% glucose, 5 mM NH_4Cl] until stationary phase was reached. At the indicated times, a portion of each culture was counted and plated on complete media to determine the number of viable cells.

fragment produced in the amplification reaction was cloned into a plasmid vector and the exact structure of the cDNA determined by sequence analysis. Two exons were positively identified, one encoding 43 amino acids and the other 368 amino acids (Figure 3). The DNA sequences corresponding to both the 5′ (AGGTAAGTAG) and the 3′ (TAG) splice junctions are in accord with the consensus splice junctions determined for *Schizosaccharomyces pombe* (Hindley and Phear, 1984).

A null allele was created by deletion of 1033 bp within the open reading frame of the $cgs1^+$ gene and insertion of the *S.cerevisiae LEU2* gene into the remaining fission yeast DNA (see Materials and methods). This construction (*cgs1::leu*) was returned to the chromosome of an h^{90}

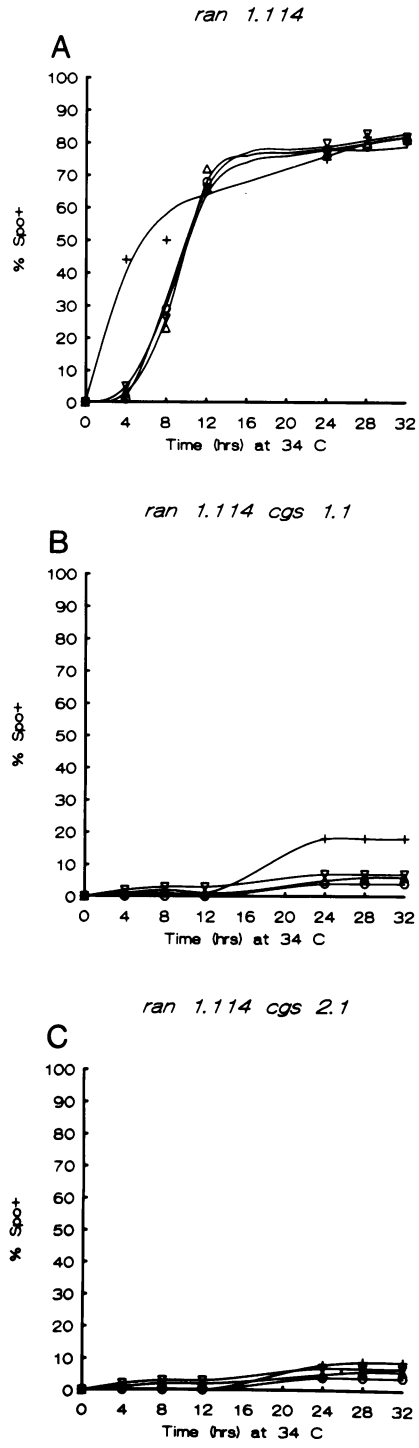

Fig. 2. Strains containing *cgs1-1* or *cgs2-1* suppress the sporulation phenotype caused by loss of *ran1-114*. Cultures of either *ran1-114* (SP514, panel **A**), *ran1-114 cgs1-1* (SP549, panel **B**) or *ran1-114 cgs2-1* (SP518, panel **C**) were grown at 25°C in PM to a density of 1×10^6 cells/ml. The cultures were then split into either (▽)PM [2% glucose, 100 mM NH_4Cl]; (○) high glucose media [4% glucose, 100 mM NH_4Cl]; (+)low glucose media [0.2% glucose, 100 mM NH_4Cl] or (+)media containing 20-fold less nitrogen [2% glucose, 5 mM NH_4Cl] and shifted to 34°C. At the indicated times, the percentage of cells that had sporulated was determined by direct microscopic observation.

leu1-32 strain of yeast by one-step gene replacement (Rothstein, 1983) of the wild type gene. Southern blotting confirmed that the wild type gene had been replaced in the stable *leu*$^+$ transformants. This strain, h^{90} leu1-32 cgs1::*leu* was sterile and failed to sporulate after protoplast fusion with a strain containing the original *cgs1-1* allele. These experiments demonstrate that we have isolated and disrupted the *cgs1*$^+$ gene. The phenotype of the *cgs1*$^+$ null allele was indistinguishable from that of the original *cgs1-1* allele.

Structure of cgs2

The *cgs2*$^+$ gene was isolated by transformation of the sporulation defective homothallic strain, SP578(h^{90} leu1-32 ade6-M210 *cgs2-2*; spo$^-$, Leu$^-$), to Leu$^+$ with a bank of wild type fission yeast DNA carried in the yeast/bacterial shuttle vector, pWH5 (Wright *et al.*, 1986). Approximately 1×10^4 transformants were examined after exposure to iodine vapors to identify spore-containing colonies. One Spo$^+$ transformant was obtained and the plasmid it contained was rescued into *E.coli*. Restriction analysis revealed that the plasmid contained a 6.3 kb *Hin*dIII fragment and further subcloning identified a 3596 bp *Pst*I fragment capable of rescuing the *cgs2* defect.

The entire nucleotide sequence of the *Pst*I fragment was determined (Figure 4). Translation of the sequence in all three reading frames revealed three large open reading frames capable of encoding peptides of 30 amino acids (Figure 4, nucleotides 1151−1240), 134 amino acids (Figure 4, nucleotides 1358−1760) and 175 amino acids (Figure 4, nucleotides 2223−2747). The 30 amino acid open reading frame contains a potential initiating methionine at position 1151 and all open reading frames identified also contained consensus acceptor and donor splice junctions. Sequence analysis also identified two *Bgl*II sites, one 28 nucleotides upstream of the putative initiating methionine (position 1151) and the other 52 nucleotides 3′ of the last predicted open reading frame. This fragment is able to rescue the sporulation defect observed in *cgs2-1* thus indicating that it contains the entire *cgs2*$^+$ gene. To determine the precise structure of the gene, a cDNA copy of the *cgs2*$^+$ transcript was synthesized using an oligonucleotide primer, P24, complimentary to the anticipated 3′ end of the gene (Figure 4, nucleotides 2721−2750; see also Materials and methods). The cDNA was amplified by polymerase chain reaction (Innis *et al.*, 1988) using primer P24 and another primer, P21, corresponding to the 5′ end of the first large open reading frame (Figure 4, 1142−1165). The fragment obtained by amplification of *cgs2*$^+$ cDNA was cloned into a plasmid vector and sequenced. This analysis revealed that *cgs2*$^+$ contained four exons: the three exons originally predicted and a fourth exon (Figure 4, nucleotides 1290−1300) capable of encoding three amino acids.

Both cgs1$^+$ *and cgs2*$^+$ *encode elements of the cAMP dependent protein kinase pathway*

The sequence of the gene products predicted to be encoded by *cgs1*$^+$ and *cgs2*$^+$ were used in a computer search of the EMBL, GenBank and PIR databases. Significant homology was found between *cgs1*$^+$ and the regulatory subunit of cAMP dependent protein kinase. The greatest degree of homology was observed between *cgs1*$^+$ and either bovine type 1 regulatory subunit or the *BCY1* gene of budding yeast (Figure 5A). The *BCY1* gene has been shown to be the regulatory subunit of cAMP dependent protein kinase in this yeast (Toda *et al.*, 1987). In common with both the *S.cerevisiae* and the bovine regulatory subunit the *cgs1*$^+$ protein product is composed of three domains: an amino-

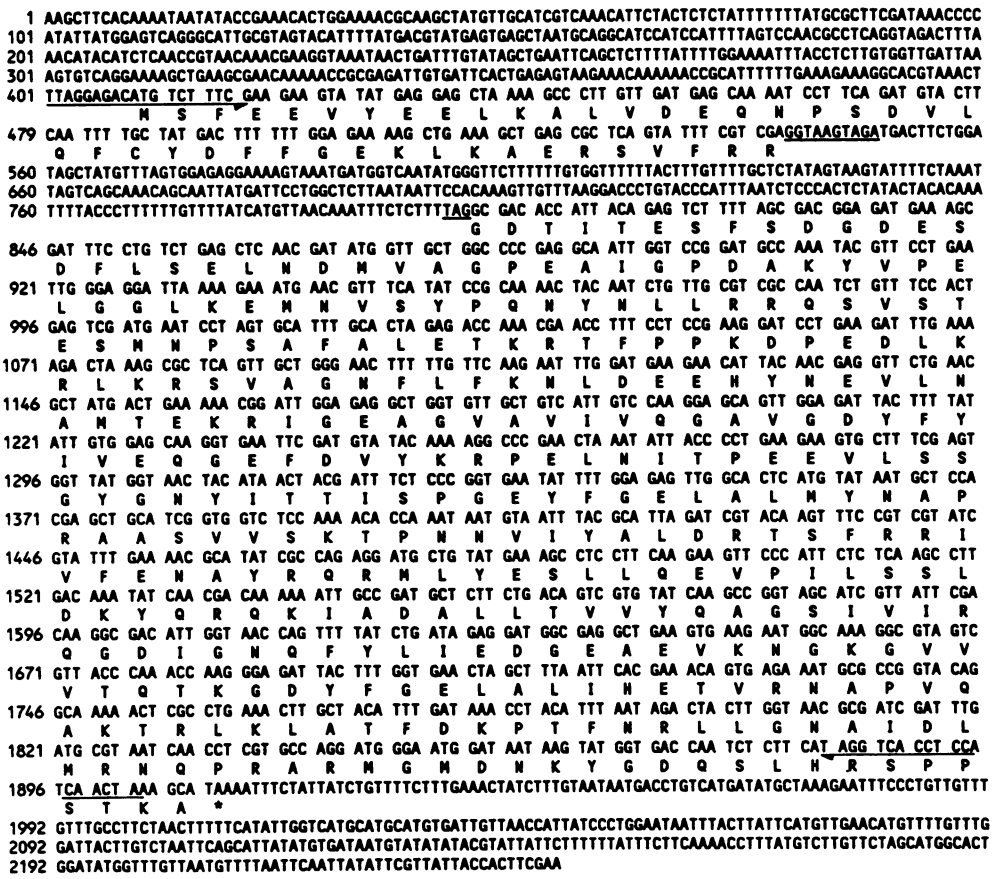

Fig. 3. Nucleotide and predicted protein sequence of the 2245 bp genomic *Hin*dIII fragment containing the *cgs1*$^+$ gene. The splice consensus sequences are underlined. The splice junctions were determined by sequence analysis of a fragment obtained by PCR amplification of a cDNA copy of the *cgs1*$^+$ gene. DNA sequence used to design oligonucleotides for PCR amplification is underlined with an arrowhead. The TAA stop codon is indicated with an asterisk.

terminal region thought to be involved in dimerization and containing the site of autophosphorylation and two regions of duplication containing the presumptive cAMP binding regions.

The assignment of *cgs1*$^+$ as the regulatory subunit of cAPK was further strengthened by biochemical studies. We examined the activity of cAPK in wild type cells and in cells containing the *cgs1-1* allele. Extract from each strain was fractionated on a MonoQ ion exchange resin (Pharmacia) and a portion of each fraction incubated with Kemptide, an eight amino acid peptide substrate of cAPK (Cheng *et al.*, 1986). Cells containing a wild type *cgs1*$^+$ gene contained cAPK activity strictly dependent on cAMP (Figure 6A). In contrast, kinase activity in a *cgs1-1* strain was independent of cAMP and the activity eluted at a salt concentration experimentally determined to be that of the free catalytic subunit rather than the holoenzyme (Figure 6B). The level of cAPK activity in fractionated *cgs1-1* extracts is lower than that present in wild type cells. It has been observed in *S.cerevisiae* that it is possible to increase the steady state level of the free cAPK catalytic subunit only with a concomitant increase in the level of the regulatory subunit (Zoller *et al.*, 1988). A number of control mechanisms are thought to exist that reduce the steady state level of cAPK activity to levels tolerable for the cell. Our results indicate that a similar homeostatic mechanism might be operative in fission yeast. From these experiments, we conclude that *cgs1*$^+$ encodes the regulatory subunit of the cAPK and that *cgs1*$^+$ is the sole cAMP regulated protein kinase in the cell able to use kemptide as a substrate.

A homology was also found between *cgs2*$^+$ and a phosphodiesterase gene from *Dictyostelium discoideum* (Figure 5B). The homology is limited to the carboxy-terminal portion of the *cgs2*$^+$ gene. A radioimmunoassay was used to determine if *cgs2*$^+$ might encode a phosphodiesterase. This was accomplished by measuring cAMP in wild type strains and in strains containing *cgs2-1*. If *cgs2*$^+$ does indeed encode a phosphodiesterase, then strains containing a mutant, loss of function allele of *cgs2*$^+$ should contain elevated levels of cAMP. Extracts prepared from wild type strains contained 2.5 pMol cAMP/mg protein, but extracts from strains containing the defective *cgs2-1* allele contained 18 pMol cAMP/mg protein or approximately a 7-fold higher amount than wild type strains (Figure 7). Taken together, these results strongly suggest that *cgs2*$^+$ does encode a cAMP phosphodiesterase.

Strains containing high levels of ran1$^+$ *kinase activity or unregulated cAPK activity are unable to induce transcription of mei2*$^+$

Several genes have been described whose functions are required for meiosis including the mating type genes, the *mei3*$^+$ gene, which is regulated by the mating type genes, and the *mei2*$^+$ gene. Loss of *mei2*$^+$ function has been shown to suppress the growth and sporulation defects associated with loss of *ran1*$^+$ (Beach *et al.*, 1985; Iino and

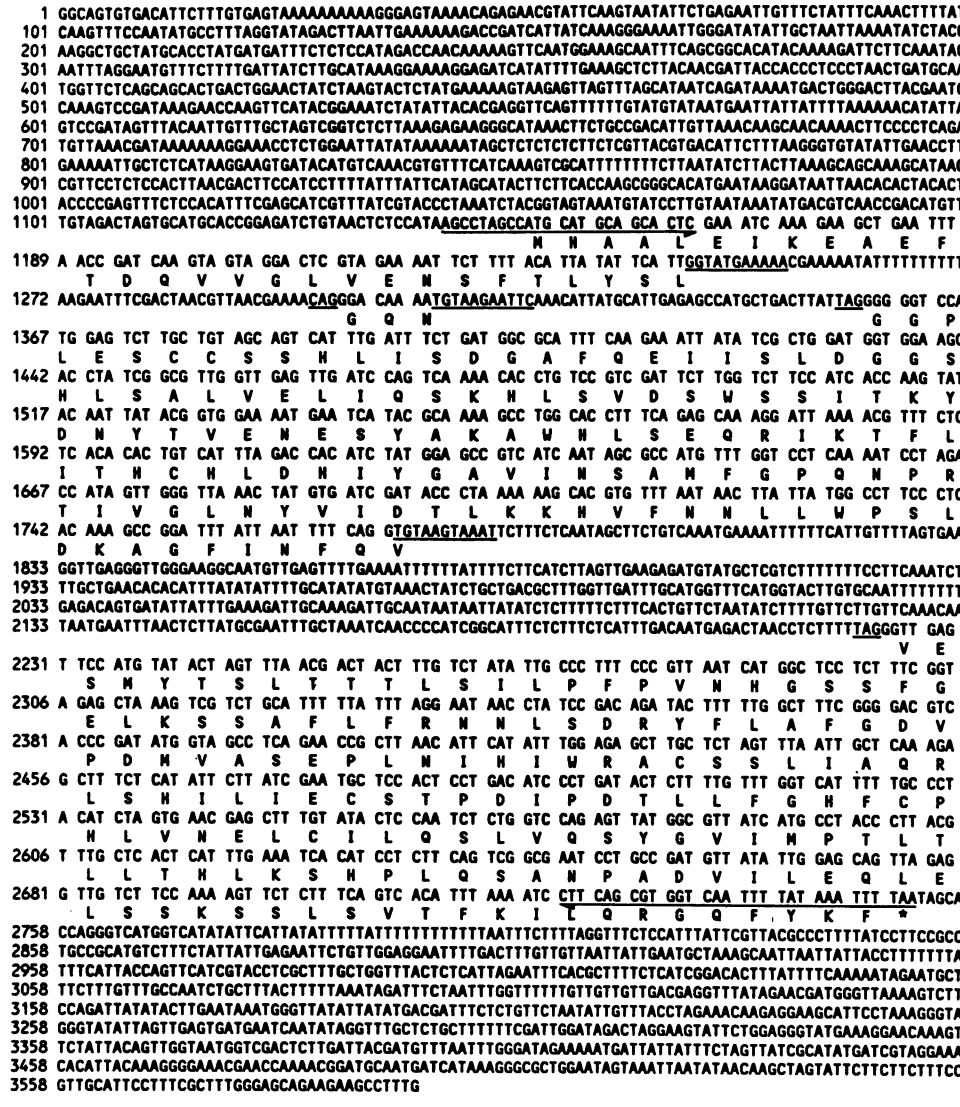

Fig. 4. Nucleotide and predicted protein sequence of the 3596 bp genomic *Pst*I fragment containing the *cgs2*$^+$ gene. The splice consensus sequences are underlined. The splice junctions were determined by sequence analysis of a fragment obtained by PCR amplification of a cDNA copy of the *cgs2*$^+$ gene. DNA sequence used to design oligonucleotides for PCR amplification is underlined with an arrowhead. The TAA stop codon is indicated with an asterisk.

Yamamoto, 1985b). Lesions in the *mei2*$^+$ gene have no effect during vegetative growth but prevent premeiotic DNA synthesis (Bresch *et al.*, 1968) which coincides with the time of meiotic commitment (Beach *et al.*, 1985). The *mei2*$^+$ transcript is induced during both vegetative growth and meiosis under conditions of nutritional starvation (Shimoda *et al.*, 1987) and inclusion of high amounts of cAMP in the media prevents accumulation of the *mei2*$^+$ transcript (Beach *et al.*, 1985; Watanabe *et al.*, 1988).

We examined the level of the *mei2*$^+$ transcript during entry into meiosis in *cgs*$^-$ strains and in strains overproducing the *ran1*$^+$ protein kinase (Figure 8). Strains constructed to produce high amounts of *ran1*$^+$ have a phenotype superficially similar to strains containing unregulated cAPK activity: they are sterile and meiotically defective (McLeod and Beach, 1988). In the experimental protocol employed, wild type cells, *cgs1-1* or *cgs2-1* containing cells, and cells containing high levels of *ran1*$^+$ kinase activity were propagated vegetatively in a medium which does not allow meiosis. The cells were transferred to a medium lacking nitrogen when they had attained a density of 5×10^6 cells/ml. Within 4 h, the *mei2*$^+$ transcript accumulates to high levels in wild type cells, but in cells unable to undergo meiosis due to either unregulated levels of either cAPK activity or *ran1*$^+$ kinase activity, no induction of *mei2*$^+$ is seen (Figure 8).

Loss of *mei2*$^+$ is sufficient to bypass the growth defect caused by loss of *ran1*$^+$. Therefore, suppression of *ran1-114* in strains containing unregulated cAPK activity might be due solely to inability to induce *mei2*$^+$. Alternatively, cAPK activity might suppress loss of *ran1-114* through regulation of multiple other gene products. We therefore asked if expression of *mei2*$^+$ in cells containing unregulated cAPK was sufficient to cause *ran1-114* containing cells to cease vegetative growth. When *mei2*$^+$ is expressed under control of a heterologous promoter in a *ran1-114* containing strain, high levels of cAMP are no longer able to suppress the growth defect caused by loss of the *ran1*$^+$ gene function (Watanabe *et al.*, 1988). Although indirect, these results indicate that the molecular basis for

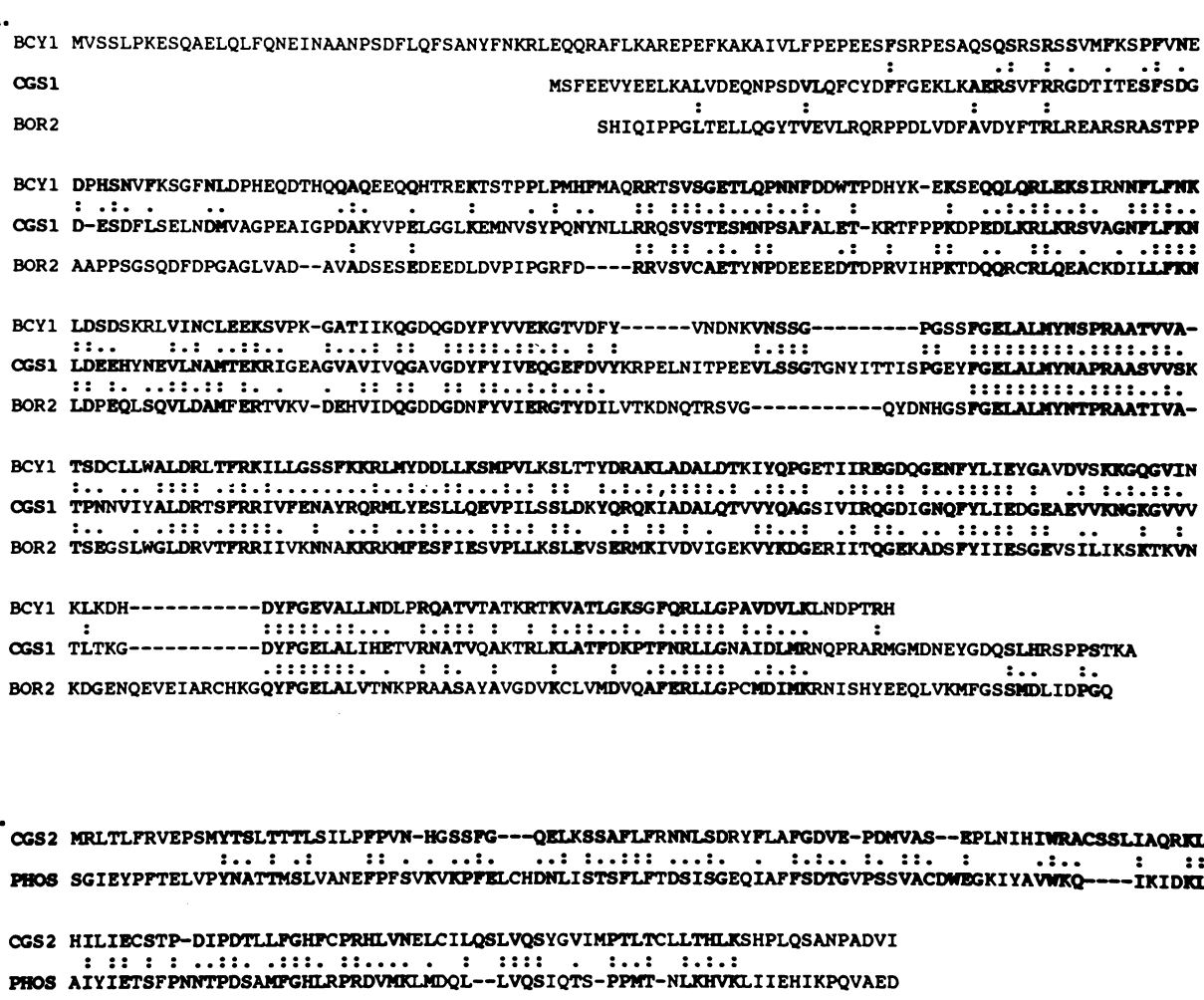

Fig. 5. Comparison of the *cgs* genes and other known proteins. (**A**) Homology between *cgs1*, *BCY1* and the bovine type 1 regulatory subunit of cAMP dependent protein kinase. Top line: *BCY1*; taken from Toda *et al.* (1987). Middle line: *cgs1*$^+$. Bottom line: Bovine RII taken from Takio *et al.* (1984). The amino acid sequences have been aligned to give the maximum homology. Identical residues are indicated with a colon; conservative amino acid changes with a period. (**B**) Homology between the cyclic nucleotide phosphoidesterase gene of *D.discoideum* (bottom line) and *cgs2*$^+$ (top line) from fission yeast. Bottom line: cAMP phosphodiesterase; taken from Lacombe *et al.* (1986). Amino acids 264−417 are shown. Top line: *cgs2*$^+$; amino acids 1−154 (Figure 4) are illustrated.

suppression of *ran1* by cAMP is the inability to induce transcription of *mei2*$^+$. We used both the *cgs1-1* and *cgs2-1*-containing strain to assess directly the effects of unregulated cAPK activity on meiosis and induction of the *mei2*$^+$ gene.

A plasmid, pMEI3.14, was constructed to contain *mei2*$^+$ under control of the constitutive ADH promoter of fission yeast (see Materials and methods). pMEI3.14 was transformed into double mutant cells containing *ran1-114* and either *cgs1-1* or *cgs2-1*. Expression of *mei2*$^+$ at the permissive temperature for *ran1-114* was not sufficient to provoke meiosis; however, when these cells were raised to the restrictive temperature for *ran1-114*, they ceased vegetative growth. In contrast, cells of the same genotype transformed with a plasmid that did not contain *mei2*$^+$ sequences were unable to undergo meiosis at either temperature (data not shown). This shows directly that unregulated cAPK activity suppresses meiosis due to loss of *ran1*$^+$ by preventing transcription of *mei2*$^+$. In addition, it indicates that regulation of meiosis by cAPK is exerted on the level of transcription of genes required prior to premeiotic DNA synthesis and not for expression of genes required later in the pathway.

Discussion

The product of the *ran1*$^+$ gene is a 52 kDa protein kinase that is required for vegetative growth and that negatively regulates sexual differentiation (Nurse, 1985; Iino and Yamamoto, 1985a). Two conditions are known to suppress the requirement for *ran1*$^+$ for vegetative growth: high levels of cAMP in the growth medium and loss of *mei2*$^+$. Previous studies have reported that agents causing unregulated cAPK activity inhibit normal meiosis and suppress the requirement of *ran1*$^+$ for vegetative growth (Beach *et al.*, 1985; Watanabe *et al.*, 1988). We have used this information to devise a genetic screen to identify genes that encode proteins regulating the activity of cAPK. In this paper we have described the isolation and characterization of two genes identified in this manner, *cgs1*$^+$ and *cgs2*$^+$. Sequence analysis and biochemical studies reveal that *cgs1*$^+$

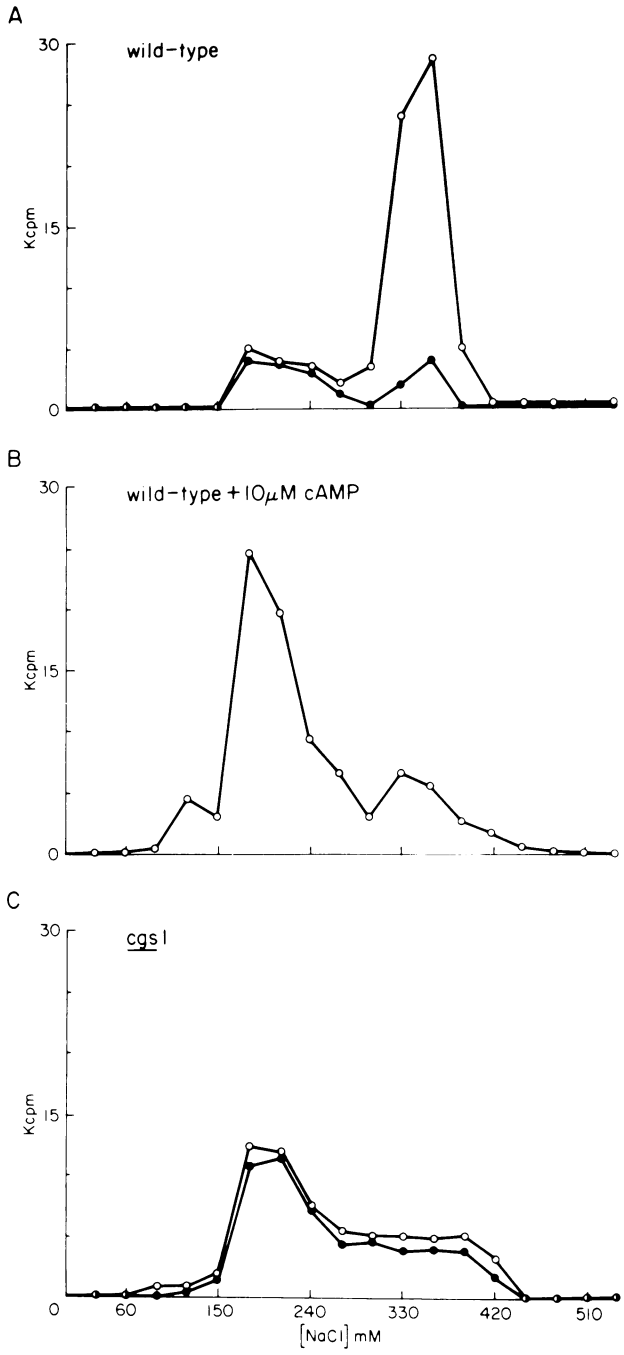

Fig. 6. cAPK activity in fractionated yeast extracts prepared from wild type and mutant strains. Extracts of wild type (panels **A** and **B**) and *cgs1-1* strains (panel **C**) were fractionated on a MonoQ (Pharmacia) column in either the absence (panels A and C) or presence of 10 mM cAMP (panel B). Portions of each fraction were incubated in a reaction mix containing [^{32}P]ATP, Kemptide and cAMP (○) or [^{32}P]ATP, Kemptide and no cAMP (●). The reactions were terminated by spotting samples on to phosphocellulose paper prior to measurement of incorporation of ATP into Kemptide using a scintillation counter.

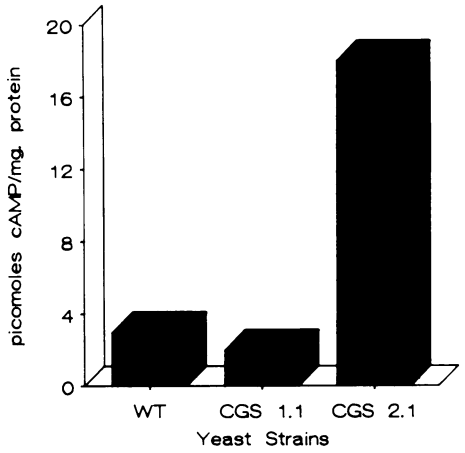

Fig. 7. Intracellular cAMP levels in wild type, *cgs1-1*, and *cgs2-1* strains. Cultures of strain SP972(h^-), SP512(h^-, *cgs1-1*) and SP489(h^-, *cgs2-1*) were grown on YEA at 30°C until a density of 1×10^7 was reached. Cell extract was prepared from each and assayed for total cell protein and amount of cAMP as detailed in Materials and methods.

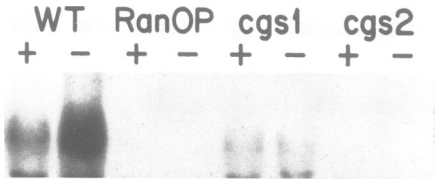

Fig. 8. Overexpression of p^{52}*ran1* protein kinase or unregulated expression of cAMP dependent protein kinase inhibits transcription of genes involved in commitment to meiosis. Cultures of wild type strain (SP253, lanes 1 and 2); a *ran1*$^+$ overproducing strain (SP618, lanes 3 and 4) a *cgs1-1* strain (SP491, lanes 5 and 6) and a *cgs2-1* strain were grown in a nitrogen-containing medium (PMA) until a density of 1×10^7 was reached. The cultures were then shifted into nitrogen-free media and incubated for 4 h. RNA was prepared from cells cultured in the presence of nitrogen (+) or from nitrogen starved cells (−) and probed for the presence of the *mei2*$^+$ transcript.

encodes the regulatory subunit of cAPK and *cgs2*$^+$ encodes a cAMP phosphodiesterase.

Normally, fission yeast exits the mitotic cell cycle under conditions of nutrient limitation and enters either the G_0 stationary phase or the sexual pathway leading to meiosis. We have determined that mutations in either *cgs1*$^+$ or *cgs2*$^+$ interfere with entry into both stationary phase and the pathway leading to sexual differentiation. Previously, we showed that cells containing high levels of *ran1*$^+$ kinase have a similar phenotype: they are completely meiotically defective (McLeod and Beach, 1988) although long term survivability studies indicate that they are able to enter stationary phase when deprived of nutrients (M.McLeod, unpublished). In this study, we have further characterized the interaction between *ran1*$^+$ protein kinase and cAPK.

Entry into meiosis is dependent on expression of the *mei2*$^+$ gene. In its absence, cells can conjugate in response to nutritional limitation but the diploid zygote formed is unable to undergo meiosis (Bresch *et al.*, 1968). In addition, the requirement of *ran1*$^+$ for vegetative growth is evident only when *mei2*$^+$ is expressed. Cells that contain unregulated cAPK activity due either to loss of *cgs1*$^+$ or *cgs2*$^+$ or to high levels of *ran1*$^+$ protein kinase are unable to induce transcription of *mei2*$^+$. Moreover, if *mei2*$^+$ is expressed under control of the ADH promoter of fission yeast, loss of either *cgs* gene is insufficient to bypass the requirement for *ran1*$^+$ during vegetative growth. Therefore, all conditions known to suppress loss of *ran1*$^+$ cause absence of a functional *mei2*$^+$ gene product. This suggests that as cells become limited for nutrients and prepare

to enter meiosis there is a concomitant decrease in the activity of certain critical enzymes, two of which are the cAPK and *ran1*$^+$ protein kinase. Inhibition of both kinases appears to be necessary for the expression of *mei2*$^+$, a critical meiotic activator.

Exit from mitosis and entry into stationary phase also requires a decrease in the activity of cAPK but not of *ran1*$^+$ kinase. Unlike entry into meiosis, entry into stationary phase is completely independent of the *mei2*$^+$ gene. Therefore, cAPK has a regulatory role in both meiotic differentiation and entry into stationary phase but *ran1*$^+$ kinase regulates entry into meiosis only. One interpretation of this result is that *ran1*$^+$ and cAPK each phosphorylate unique substrates but also share a set of common substrates, one or more of which acts in both entry into the stationary phase and meiosis. The preferred phosphorylation site of cAPK is contained within Kemptide which is not a substrate of *ran1*$^+$ kinase *in vitro* and thus it is unlikely that both kinases phosphorylate the same site *in vivo*. However, it has been proposed that activation of a substrate by phosphorylation is sometimes a multi-step process and that phosphorylated sites interact cooperatively to potentiate each other (Blackshear *et al.*, 1988). We anticipate that these as yet unidentified substrates either directly or indirectly modulate transcription of genes required for induction of either the stationary phase state or meiosis (Nielson and Egel, 1990). Alternatively, *ran1*$^+$ and cAPK could be components of a protein kinase cascade that regulates entry into meiosis or into stationary phase. For instance, the genetic data are consistent with a model in which *ran1*$^+$ could positively regulate cAPK activity either directly or indirectly by phosphorylation.

It is intriguing that mutations that lead to unregulated cAPK activity cause cells to become elongated, particularly as nutrients become depleted. Normally, poor nutrient conditions cause advancement into mitosis which results in shortened cells (Fantes and Nurse, 1977). The observation that *cgs*$^-$ cells do not appear to exhibit a stimulated rate of cell division as stationary phase is approached indicates that the cAPK might play a role in the nutritional regulation of mitotic size control.

The response of fission yeast to nutritional limitation is complex. Cells increase their rate of cell division, divide at a small size and finally enter stationary phase. In stationary phase, fission yeast become resistant to heat shock and exhibit long term survivability, as do mammalian cells which have entered G_0. In yeast, nutritional limitation is also required for sexual differentiation. The *ran1*$^+$ kinase controls sexual differentiation, but at least one other regulatory enzyme controlling these events in yeast appears to be cAPK activity.

Materials and methods

Yeast strains and growth media

All strains of *S.pombe* used in this study were derived from the original isolates h^{90}, h^{-s} and h^{+N} introduced by U.Leupold. Strains carrying the *ran1-114* allele (Iino and Yamamoto, 1985) were derived from those provided initially by M.Yamamoto. The strains used were as follows: SP66 h^{90} *leu1-32 ade6-M216*; SP259 h^{90} ran1-114 ade6-M216; SP253 h^{90} *ade6-M210*; SP489 h^{-s} *cgs2-1*; SP491 h^{90} *leu1-32 cgs1-1 ade6-M216*; SP512 h^{-s} *cgs1-1*; SP514 h^{-s} *cgs1-1 ade6-M216*; SP518 h^{-s} *cgs2-1 ran1-114 ade6-M216*; SP519 h^{90} *cgs2-1 leu1-32 ade6-M210*; SP549 h^{-s} *cgs1-1 ran1-114 ade6-M216*; SP972 h^{-s}.

Yeasts were cultured either in rich media (YEA; 0.5% yeast extract, 3% glucose, 0.075 mg/ml 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.

Extragenic suppressors of ran1-114

SP259 was grown at 25°C until cells attained a density of 1×10^7/ml. Ethylmethanesulfonate (Kodak) was added to 2% and the cells were incubated for 4 h. The mutagenized cells were grown at 25°C and replica plated to YEA at 34°C to identify cells able to grow at the high temperature. A total of 412 suppressors were identified and 365 of these contained a defect in the *mei2*$^+$ gene. Of the remaining 47 colonies, three (SP484, SP515 and SP488) contained elongated cells. Each *cgs* mutant was obtained free of the *ran1-114* mutation and all were found to be sterile. Pairwise crosses of each revealed that two complementation groups were represented.

Measurement of survival after entry into stationary phase

The indicated strains were grown in the appropriate media (see Figure 1B) at 32°C to a density of 1×10^7/ml. The cultures were maintained at this temperature and at daily intervals, a portion of each was removed, diluted and plated onto YEA plates for cultivation at 32°C. The colonies formed were counted manually after 3 days.

Cytology

The appropriate *S.pombe* strains were stained with diamidinophenylindole (DAPI, Sigma) according to the method of Marks and Hyams (1985).

Cloning of cgs1$^+$ *and cgs2*$^+$

Schizosaccharomyces pombe strains SP491 (h^{90} *leu1-32 cgs1-1 ade6-M210*) or SP519 (h^{90} *leu1-32 cgs2-1 ade6-M210*) were transformed as described by Beach and Nurse (1981) with gene banks generously constructed and provided by Dr Paul Young (Queen's University, Kingston, Ontario). Transformants were selected on PMA + sorbitol plates and incubated at 32°C. The plates were inverted over iodine crystals to identify colonies that had acquired the ability to sporulate. Plasmids were recovered from *S.pombe* transformants into *E.coli* strain JA226 as described (Beach *et al.*, 1982). Each plasmid was retested following this procedure to determine that it contained sequences capable of complementing the *cgs spo*$^-$ phenotype. Two plasmids, each containing non-overlapping fragments, were able to complement *cgs1-1*: pCGS1-1 and pcgs1.X. One of these, pcgs1.X, was not linked to *cgs1-1* when it was integrated by homologous recombination into the chromosome. Only pCGS1.1 was linked to *cgs1-1* and, due to the small size of the genomic fragment on the plasmid, was directly sequenced. The plasmid pcgs2.1 was able to complement *cgs2.1* and was found to be linked to the gene. It was subcloned until a 3596 bp fragment was identified that was able to complement the sporulation defect caused by loss of *cgs2-1*.

Plasmid constructions

pMEI2.14 was constructed by insertion of a DNA fragment containing the *mei2*$^+$ gene into the *S.pombe* expression vector pART3 (McLeod and Beach, 1988). The *mei2*$^+$ gene was isolated as a fragment that could rescue the sporulation defect of a *mei2* strain and oligonucleotide mutagenesis was used to insert an *NDE1* site at the initiating ATG of the gene (Watanabe *et al.*, 1988). The complete *mei2*$^+$ gene was then ligated into the pART3 expression vector as an *Nde*I or *Sal*I fragment.

Oligonucleotides used in this study

Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems 380A synthesizer. Their sequence is given below. Primers P1 and P21 correspond to coding strand DNA sequence contained within the first exon of *cgs1*$^+$ and *cgs2*$^+$ respectively. Primers P4 and P24 are complimentary to non-coding strand DNA sequence contained within the predicted carboxy-terminus of *cgs1*$^+$ and *cgs2*$^+$ respectively. The position of each oligonucleotide within *cgs1*$^+$ is illustrated in Figure 4; those contained within *cgs2*$^+$ are illustrated in Figure 5.

P1 5'CGTAAAGCTTTAGGAGACATGTCTTTCGAAG3'
P4 5'TATGCTTTAAGCTTGATGGAGGTGACCTATGA3'
P24 5'TTAAAATTTATAAAATTGACCACGCTGAAG3'
P21 5'AGCCTAGCCATGCATGCAGCACTC3'

Nucleotide sequence determination

The DNA sequence of *cgs1*$^+$ and of *cgs2*$^+$ was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Single-stranded DNA template was prepared from pUC118 plasmids carrying either a *Hin*dIII insert of *cgs1*$^+$ or of *cgs2*$^+$. pUC118 plasmids are derived from pUC18 by insertion of a 300 bp fragment from the intergenic region of M13. The plasmid was obtained in single-stranded form

following superinfection of plasmid-containing *E.coli* with phage M13KO7 (J.Vieira, personal communication).

To obtain a series of overlapping clones for sequencing, the *Hin*dIII fragment carrying $cgs1^+$ was filled in using Klenow polymerase and the blunt-ended fragment was inserted into the *Sma*I site of pUC118 in both possible orientations. Unidirectional deletions were constructed according to the method of Henikoff (1987).

cDNA synthesis and amplification

RNA was isolated as described below and 10 μg annealed with 20 pM P4 primer. cDNA was synthesized by extension of this primer using reverse transcriptase (BRL) exactly according to the manufacturer's instructions. After hydrolysis using NaOH and EtOH precipitation, the second strand was synthesized using 2 U TAQ polymerase (Cetus) and 30 cycles of co-amplification. The sequences corresponding to the primers used are indicated in Figures 4 and 5. Temperatures were 94°C for denaturation, 55°C for polymerization and 72°C for annealing. The fragments obtained were re-amplified using the same conditions. To obtain flush end molecules, the amplified DNA was EtOH precipitated and treated with Klenow polymerase. These were ligated into *Sma*I digested pUC118 and transformed into DH5 (BRL).

RNA isolation

20 ml cells (SP972) were harvested at the appropriate stage of growth (see text) and washed with TE (10 mM Tris, pH 7.4, 1 mM EDTA). The pelleted cells were resuspended in 0.1 ml 10 mM Tris pH 7.4, 1 mM EDTA and 0.1 ml phenol. Glass beads (0.45 microns) were added to the meniscus. Cells were broken by vortexing for 5 min after which TE +0.1% sodium lauryl sulfate was added. Unbroken cells were pelleted at 12 000 *g* and the supernatant was extracted twice in phenol. RNA was precipitated in 0.25 M LiCl and 70% EtOH and stored at −70°C until it was used.

Northern analysis

Total RNA (20 μg) was fractionated on 1.5% formaldehyde agarose gels as described in Maniatis *et al.* (1982) and transferred to Hybond-N+ exactly as described (Amersham). A probe to $mei2^+$ was prepared by random priming using a kit from Boehringer Mannheim. Hybridizations were performed using 1×10^7 c.p.m. ^{32}P-labeled probe in 4 × SSC, 5 × Denhardts, 25 mM $NaHPO_4$, pH 7.0, 50% formamide and 0.25 mg/ml salmon sperm DNA with incubation in a shaking water bath at 42°C for 12 h (Maniatis *et al.*, 1982). Filters were washed at room temperature in two changes of 2 × SSC, 0.1% SDS followed by four washes in 0.1 × SSC, 0.1% SDS. The final wash was at 65°C in 0.1 × SSC, 0.1% SDS. The filters were dried and exposed to photographic film for 4 h to overnight.

Measurement of cAPK activity

Cultures of SP972 or SP512 were grown at 30°C in YEA to a density of 5×10^6/ml. Cells were harvested by centrifugation and resuspended in buffer A (50 mM Tris pH 7.4, 2 mM EDTA, 1 mM benzamadine HCl, 1 mM PMSF, 1 mM DTT) and broken using glass beads. The extract was centrifuged at 13 000 *g* for 20 min and filtered through a 0.2 micron HAWP filter (Millipore). Protein (25 mg) was applied to a MonoQ5/5 (Pharmacia) column and eluted using a gradient from buffer A to buffer A, 1 M NaCl. A total of 50 0.5 ml fractions were collected. Kinase reactions were performed using 10 μl extract was described (Zoller *et al.*, 1988).

Measurement of cAMP

Cultures of SP972, SP512 and SP486 were grown at 30°C in PMA to a density of 5×10^6/ml. Cells were collected on filters and processed and cAMP was measured by radioimmunoassay exactly as described (Field *et al.*, 1990).

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