The EMBO Journal vol.9 no.5 pp.1401 – 1406, 1990

# The *pat1* protein kinase controls transcription of the mating-type genes in fission yeast

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Communicated by P.Nurse

**The developmental programme of fission yeast brings about a transition from mitotic cell division to the dormant state of ascospores. In response to nitrogen starvation, two cells of opposite mating type conjugate to form a diploid zygote, which then undergoes meiosis and sporulation. This differentiation process is characterized by a transcriptional induction of the mating-type genes. Conjugation can also be induced in *pat1-ts* mutants by a shift to a semi-permissive temperature. The *pat1* gene encodes a protein kinase, which also functions further downstream in the developmental pathway controlling entry into meiosis. We have analysed transcriptional induction of mating-type genes in various strains—with and without a *pat1-ts* allele. In wild-type cells of *P*-mating type derepression occurs in two rounds. First, the *mat1-Pc* gene is induced in response to nitrogen starvation. Mutants in the *map1* gene are defective in this process. In the following step the *mat1-Pm* gene is expressed in response to a pheromone signal generated by cells of *M* mating type. Both these controls are derepressed in the *pat1-ts* mutant at semi-permissive temperature. Previous work has established that expression of the mating-type genes in the zygote leads to complete loss of *pat1* protein kinase activity causing entry into meiosis. Thus, *pat1* can promote its own inactivation. We suggest a model according to which a stepwise inactivation of *pat1* leads to sequential derepression of the processes of conjugation and meiosis.**

*Key words:* conjugation/mating pheromones/meiosis/*pat1* protein kinase/*Schizosaccharomyces pombe*

## Introduction

The fission yeast *Schizosaccharomyces pombe* provides an attractive model system for studying eukaryote growth control and differentiation at the unicellular level. It can be propagated in haploid as well as diploid forms, and is readily investigated by the powerful combination of genetic and molecular analysis (Moreno *et al.*, 1990). As long as nutrients are sufficient, cells grow by haploid mitosis. Depletion for a nitrogen source causes cells to differentiate (Egel, 1971). First, two cells conjugate to form a diploid zygote which then directly undergoes meiosis and sporulation (Leupold, 1950). It is possible to make the zygotes resume mitosis as diploid cells at low frequency if they are transferred to fresh growth medium (Egel, 1973a). Once such diploid cells encounter nitrogen starvation, they will enter meiosis without a preceding round of conjugation.

The conjugation process is restricted to pairs of cells having opposite mating types, *P* (for Plus) and *M* (for Minus). A haploid cell exhibits the *P* or *M* mating type depending on whether the *P* or *M* allele is present at a single genetic locus called *mat1*. The homothallic wild-type strain ($h^{90}$) is a mixture of *P* and *M* cells, because the vegetatively growing cells switch from one mating type to the other once every few cell divisions (Egel, 1977; Miyata and Miyata, 1981) by mobilizing silent information stored at *mat2* and *mat3* (Egel and Gutz, 1981; Beach, 1983).

The *mat1-M* locus contains two divergently transcribed genes (called *mat1-Mc* and *mat1-Mm*), and, similarly, the *mat1-P* locus contains two divergently transcribed genes (called *mat1-Pc* and *mat1-Pm*) (Kelly *et al.*, 1988). The *mat1-Mm* and *mat1-Pm* genes are also referred to as *mat1-Mi* and *mat1-Pi*, but here we use the *m*-suffices to indicate the meiotic functions of these genes; see below. The *mat1-Mc* gene alone is sufficient for *M* cell conjugation, and the *mat1-Pc* gene is sufficient for *P* cell conjugation. In mitotic *M* and *P* cells these two genes are transcribed at a low level, whereas the *mat1-Mm* and *mat1-Pm* transcripts are undetectable. When cells experience nitrogen starvation and start to differentiate, the four *mat1* transcripts are all strongly induced (Kelly *et al.*, 1988).

In addition to specifying cellular mating type, the *mat1* locus also controls entry into meiosis (Egel, 1973a). Simultaneous expression of all four *mat1* genes in an *M/P* diploid cell under nitrogen starvation allows transcription of an unlinked gene, *mei3* (McLeod *et al.*, 1987). The *mei3* gene product, $p21^{mei3}$, is a meiotic inducer. Forced expression of the *mei3* gene in vegetatively growing cells causes meiosis. The function of $p21^{mei3}$ is to inhibit a protein kinase, $p52^{pat1}$, which is a repressor of meiosis. Purified $p21^{mei3}$ specifically abolishes the kinase activity of purified $p52^{pat1}$ *in vitro* (McLeod and Beach, 1986, 1988). The $p52^{pat1}$ protein is encoded by the *pat1* gene (also referred to as *ran1*), which is essential for mitotic growth. Temperature-sensitive *pat1* mutants undergo meiosis at the restrictive temperature, irrespective of the *mat1* configuration and the nutritional conditions (Nurse, 1985; Iino and Yamamoto, 1985a). The *pat1* gene product presumably phosphorylates at least one protein, which causes meiosis when dephosphorylated. A candidate for such a protein is the *mei2* gene product. The induction of meiosis in temperature-sensitive *pat1* alleles is completely suppressed by *mei2* mutations (Beach *et al.*, 1985; Iino and Yamamoto, 1985b). The $mei2^{+}$ function is the furthest downstream control point identified before cells become committed to meiosis (Egel and Egel-Mitani, 1974; Watanabe *et al.*, 1988).

The $p52^{pat1}$ protein kinase also functions as a repressor of conjugation. Mating between *P* and *M* cells in the presence of a nitrogen source is induced when the temperature is raised to a semi-permissive level in temperature-sensitive *pat1* mutants (Nurse, 1985; Beach *et al.*, 1985). In the present study we show that such partial inactivation of *pat1* induces

transcription of the *mat1* genes. Hence, this process mimics conjugation in wild-type cells starved for a nitrogen source. Based on genetic data we suggest a model for differentiation with inactivation of $p52^{pat1}$ occurring in three steps.

## Results

### *P cell mutants*

Several mutants have been characterized as being defective in one of the two mating types. The *map1*$^+$ function is required for *P* cell conjugation (Egel, 1973b). Thus, when *map1* mutations are present in a homothallic background, cells switch between *M* mating type and sterility. The *P* mating defect occurs very early in conjugation; none of the morphological changes associated with mating are observed. The *P*-sterile cells do not secrete the *P* mating pheromone, and do not respond to the pheromone secreted by *M* cells (Leupold *et al.*, 1989a). This suggests that such mutants are defective in the process of sexual induction upon nitrogen starvation. Since this process is characterized by induction of the *mat1-Pc* transcript, we tested whether this is abolished in *map1* mutants (Figure 1a). The homothallic $h^{90}$ *fus1* strain and a heterothallic $h^+$ strain of *P* mating type both induce transcription of the *mat1-Pc* gene after nitrogen starvation. In this experiment, the *fus1* mutation (Bresch *et al.*, 1968) was included in the homothallic strain to prevent zygote formation. The homothallic $h^{90}$ *map1* strain, however, fails to raise this transcript above the low basal level of mitotic cells.

Mutants in the *mat1-Pc* gene are also defective in *P*-cell induction (Meade and Gutz, 1976; Leupold *et al.*, 1989a) and have a phenotype identical to *map1* mutants (see below). As shown in Figure 1a, an $h^{90}$ *mat1-Pc*-161 strain also fails to induce the *mat1-Pc* transcript upon nitrogen starvation, indicating that the *mat1-Pc* gene is positively autoregulated. We conclude that the basal level of the *mat1-Pc* gene product together with the *map1*$^+$ function is required for high-level synthesis of the *mat1-Pc* transcript during differentiation. In the absence of induction no sexual activities are initiated in *P* cells.

### *Meiotic functions*

The role of the various *P* mating functions in the control of meiosis appears complex. In addition to controlling *P* cell conjugation the *mat1-Pc* and *map1* gene products are required for entry into meiosis. Hence, diploid *mat1-M*$^+$/*mat1-Pc*$^-$ cells as well as diploid *P/M* cells homozygous for the *map1* mutation fail to undergo meiosis (Egel, 1973b; Meade and Gutz, 1976). Furthermore, the *mat1-Pm* gene, which has no function in conjugation, is required for entry into meiosis. This gene is not at all expressed in mitotic cells, but is activated during sexual induction. The experiment described in Figure 1b shows that nitrogen-starved $h^{90}$ *map1* and $h^{90}$ *mat1-Pc* mutants do not express the *mat1-Pm* gene. Thus, the failure of these mutants to become sexually induced results in the inability to transcribe the *mat1-Pm* gene in response to nitrogen starvation. This suggests that the meiotic defect of *map1* and *mat1-Pc* mutants is indirect and due to lack of the *mat1-Pm* gene product. Indeed, similar to haploid strains, diploid *map1* and *mat1-Pc* strains fail to synthesize *mat1-Pm* RNA (Fujioka and Shimoda, 1989; O.Nielsen, unpublished data).

A heterothallic $h^+$ strain also fails to induce the *mat1-Pm* gene upon nitrogen starvation (Figure 1b). This strain induces the *mat1-Pc* transcript (Figure 1a) and such cells can respond to *M* factor (Leupold, 1987), indicating that sexual induction does occur. In the $h^{90}$ *fus1* strain, where *M* cells are present, the *mat1-Pm* gene is transcribed (Figure 1b) even though cell fusion is prevented by the *fus1* mutation. This suggests that the *mat1-Pm* transcript is synthesized in response to the *M* factor pheromone.

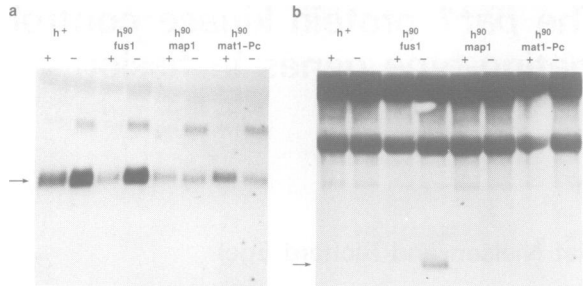


**Fig. 1.** Northern blot analysis of the effects of nitrogen starvation in various yeast strains on expression of the *mat1-P* genes. For each of the strains tested the first lane (+) contains RNA from mitotic cells and the second lane (−) from nitrogen starved cells. The following yeast strains were tested: $h^+$ (Eg281), $h^{90}$ *fus1* (Eg392), $h^{90}$ *map1* (Eg393) and $h^{90}$ *mat1-Pc* (Eg410). (**a**) Expression of the 750 bp *mat1-Pc* transcript (arrowed). A larger transcript of unknown identity which is recognized by the probe is induced by nitrogen starvation in all the four strains. (**b**) Expression of the 550 bp *mat1-Pm* transcript (arrowed). The two large bands seen in all lanes represent unspecific hybridization to 18S and 25S ribosomal RNAs (here serving as an internal standard, although these bands could have been removed by stringent washing).

### *pat1 controls the mat1 genes*

The *pat1* protein kinase is a repressor of both conjugation and meiosis. When the temperature-sensitive *pat1*-114 mutant is shifted from the permissive temperature of 23°C to a semi-permissive temperature of 30°C, conjugation between *P* and *M* cells is induced in nitrogen-rich medium (Beach *et al.*, 1985). The experiment described in Figure 2 shows that such conjugation driven by partial inactivation of *pat1* function mimics wild-type conjugation caused by nitrogen starvation. A strong induction of the *mat1-Pc* transcript (Figure 2a) and the *mat1-Mc* transcript (Figure 2b) is observed when the temperature is raised in an $h^{90}$ *pat1*-114 strain. This induction is not seen in an $h^{90}$ control strain (Figure 2a). Therefore, active *pat1* kinase represses transcription of *mat1-Pc* and *mat1-Mc*, which presumably prevents conjugation in mitotic cells. Partial inactivation of the *pat1*-114 allele also induces transcription of the *mat1-Pm* gene (Figure 2c).

### *Gene interactions*

The *map1* and *mat1-Pc* gene products are necessary for conjugation and meiosis in *P* cells. Are these functions also required in differentiation driven by inactivation of *pat1-ts*? To address this question, homothallic *map1 pat1* and *mat1-Pc pat1* double mutants were constructed (Eg401 and Eg434; Table I). When the *pat1*-114 gene product was completely inactivated in these double mutants by shifting the temperature from 23° to 35°C, the cells underwent haploid sporulation. This is the same phenotype as that exhibited by *pat1*-114 single mutants (Iino and Yamamoto, 1985a), showing that the meiotic defects of *map1* and

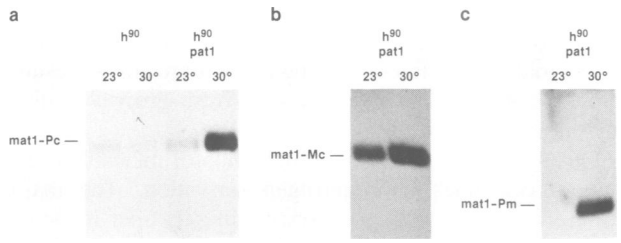


**Fig. 2.** Effect of temperature shift-up (°C) on expression of *mat1* genes in *pat1* mutant strains. (**a**) Expression of the *mat1-Pc* gene in the $h^{90}$ wild-type strain (Eg282) and the $h^{90}$ *pat1*-114 mutant strain (Eg397). (**b**) Expression of *mat1-Mc* in strain Eg397. (**c**) Expression of *mat1-Pm* in Eg397.

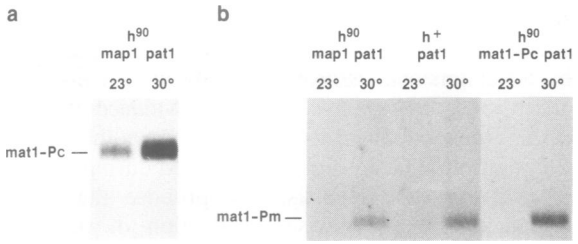


**Fig. 3.** Induction of the *mat1-P* transcripts at 30°C in double mutant strains. (**a**) Induction of the *mat1-Pc* transcript after temperature shift-up in the $h^{90}$ *map1 pat1* strain Eg401. (**b**) Expression of *mat1-Pm* at 30°C in the following strains: $h^{90}$ *map1 pat1* (Eg401), $h^{+}$ *pat1* (Eg423) and $h^{90}$ *mat1-Pc pat1* (Eg434).

**Table I.** Experimental strains of *Schizosaccharomyces pombe*

| Strain | Genotype | Source |
|---|---|---|
| Eg281 | *mat1-P mat2,3*-del::*LEU2* | Klar and Miglio (1986) |
| Eg282 | $h^{90}$ wild type | Leupold (1950) |
| Eg392 | $h^{90}$ *fus1*-B20 | Bresch *et al.* (1968) |
| Eg393 | $h^{90}$ *map1*-A83 | Egel (1973b) |
| Eg397 | $h^{90}$ *pat1*-114 | Iino and Yamamoto (1985a) |
| Eg401 | $h^{90}$ *map1*-A83 *pat1*-114 | this study |
| Eg410 | $h^{90}$ *mat1-Pc*-161 | Meade and Gutz (1976) |
| Eg423 | *mat1-P mat2,3*-del::*LEU2 pat1*-114 | this study |
| Eg434 | $h^{90}$ *mat1-Pc*-161 *pat1*-114 | this study |

*mat1-Pc* mutants can be suppressed by full *pat1* inactivation. However, when *pat1*-114 repression of conjugation was relieved by shifting the temperature from 23° to 30°C, cells of these double mutant strains failed to conjugate altogether. Control cells of a homothallic *pat1*-114 strain (Eg397, Table I) showed marked conjugation after 8 h at 30°C. This experiment shows that the $map1^{+}$ and $mat1\text{-}Pc^{+}$ functions are indeed required in conjugation driven by partial *pat1*-114 inactivation.

### *Transcriptional activation of mat1-Pc*

In the previous sections we have shown that the *pat1* protein kinase is required to repress transcription of the *mat1-P* genes, and that the $map1^{+}$ and $mat1\text{-}Pc^{+}$ functions are necessary for induction of these transcripts after nitrogen starvation. Furthermore, *map1 pat1* and *mat1-Pc pat1* double mutants fail to conjugate when the *pat1* repression of mating is relieved. We then determined whether the induction of *mat1-Pc* caused by inactivation of *pat1* is still *map1* dependent. As shown in Figure 3a this is not the case. The $h^{90}$ *map1 pat1*-114 strain induces the *mat1-Pc* transcript at 30°C as does the homothallic *pat1*-114 strain (Figure 2a). Hence, the $map1^{+}$ function is required for conjugation at some other step in addition to its requirement for induction of the *mat1-Pc* transcript.

### *Repression of mat1-Pm*

In wild-type strains, *map1* and *mat1-Pc* are required for activation of the *mat1-Pm* gene. Is *pat1* also involved in this regulation? As shown in Figure 3b, *map1 pat1* and *mat1-Pc pat1* double mutants induce transcription of *mat1-Pm* at 30°C. Therefore, the requirement of the $map1^{+}/mat1\text{-}Pc^{+}$ function for *mat1-Pm* transcription is bypassed when *pat1*-114 is partially inactivated. Even a heterothallic $h^{+}$ *pat1*-114 strain expresses the *mat1-Pm* gene when the temperature is raised to 30°C (Figure 3b). Thus, *pat1* directly controls transcription of *mat1-Pm* in addition to the indirect control exerted through *map1* and *mat1-Pc*. In $pat1^{+}$ strains a pheromone signal is required in order to relieve this repression.

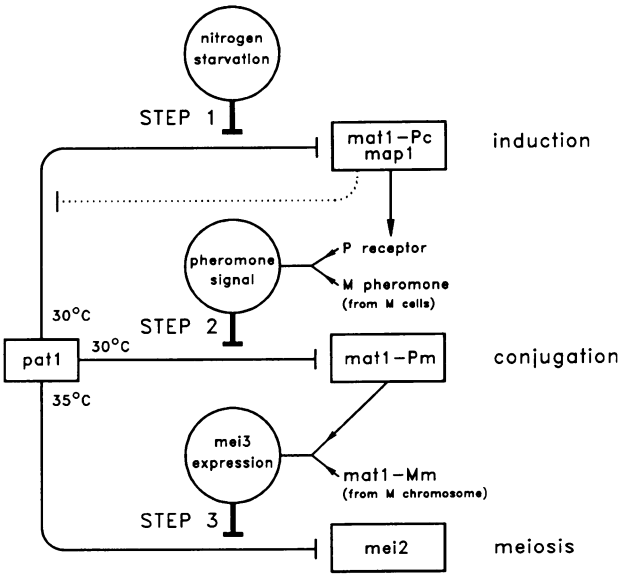


**Fig. 4.** A model for differentiation in cells of *P* mating type by gradual inactivation of the *pat1* protein kinase. The signal of nitrogen starvation induces *P*-cell mating activities. This process is characterized by transcriptional induction of the *mat1-Pc* and *map1* genes, and requires inactivation of the *pat1* protein kinase. In *pat1*-114 mutant (ts) strains the thermal inactivation at 30°C is sufficient for transcriptional induction to occur. In $pat1^{+}$ strains the $map1^{+}$ and $mat1\text{-}Pc^{+}$ functions are required to further inactivate *pat1* function (dotted line). When the sexually induced *P* cell encounters a cell of *M*-mating type, the signal triggered by the *M* pheromone will relieve *pat1* repression of *mat1-Pm* transcription. Other gene functions (such as *fus1*) specifically required for conjugation are activated at this point. Finally, upon successful conjugation, the joint presence of both the *mat1-Pm* and *mat1-Mm* gene products in the zygote will induce transcription of the *mei3* gene. This fully inactivates the *pat1* protein kinase, and triggers meiosis and sporulation. Lines ending in a bar symbolize repression of function.

## Discussion

### *A cascade of pat1 inactivation*

Sexual differentiation in *S.pombe* is an example of a highly regulated developmental process. In response to nutritional deprivation the cells are induced to form ascospores by

means of the co-ordinated processes of conjugation and meiosis. The *pat1* protein kinase functions as a master switch in this developmental pathway. By analysing a temperature-sensitive mutant, we have provided evidence that *pat1* represses conjugation and entry into meiosis at three points. These points are successively derepressed during differentiation of wild-type *P* cells. We propose that this is accomplished by a stepwise inactivation of the $pat1^+$ protein kinase (Figure 4). The first inactivation occurs when the cells encounter nitrogen starvation (step 1). This induces the *mat1-Pc* gene which together with the $map1^+$ function is necessary to set up the *P*-specific pheromone response system.

The simplest model compatible with our observations on induction of the *mat1-Pc* gene is that the *pat1* kinase and the *map1*/*mat1-Pc* function modulate the transcription level of *mat1-Pc* in a positive feedback loop. In mitotic cells, active *pat1* allows synthesis of only a low basal level of *mat1-Pc* RNA; transcription is strongly induced when the *pat1* kinase is inactivated. If the inactivation is caused by nitrogen starvation in a *pat1* strain, further inactivation by the *map1*/*mat1-Pc* function is necessary for full activation of *mat1-Pc* (Figure 4, dotted line). In a *pat1*-114 strain, on the other hand, the thermal inactivation of *pat1* at 30°C is sufficient for induction of *mat1-Pc* even in the absence of the *map1*/*mat1-Pc* functions. The *map1* gene appears to be regulated in the same manner as *mat1-Pc* (O.Nielsen, unpublished data).

When the nitrogen-starved cell finds a mating partner, the pheromone signal triggered by the *M*-factor relieves the second control point (step 2). This induces transcription of the *mat1-Pm* gene. Gene functions that are required specifically for cell conjugation are also expected to be activated at this point. Indeed, we have characterized the conjugation-specific gene *fus1*, which is regulated in a manner similar to *mat1-Pm* (D.Weilguny and R.Egel, unpublished data).

The final inactivation event is brought about only after successful zygote formation when meiosis is appropriate (step 3). Together with the *mat1-M* function, the *mat1-Pm* gene product activates the *mei3* gene in the zygote (McLeod *et al.*, 1987), and this fully inactivates the *pat1* kinase (McLeod and Beach, 1988). Derepression of this third control point activates the *mei2* protein (Beach *et al.*, 1985; Iino and Yamamoto, 1985b) which triggers meiosis.

According to this scheme, the *pat1* protein kinase is taking part in its own down-regulation. Partial inactivation of *pat1* induces the pheromone communication system. When the cell receives a pheromone signal *pat1* is further inactivated, leading to expression of the *mat1-Pm* gene and of functions required for conjugation. Upon zygote formation the *mat1-Pm* gene product (together with *mat1-Mm*) then allows synthesis of the *mei3* protein which fully inactivates *pat1*. Thus, partial inactivation of the *pat1* kinase is promoting its own full inactivation.

Conceivably, the chain of inactivation occurring in *M*-cells is analogous, that is the *mat1-Mc* gene is necessary for pheromone communication during conjugation and this activates the *mat1-Mm* gene. Since the *mat1-P* and *mat1-M* gene products are already made in the prezygotic cells, the zygotes are primed to synthesize the *mei3* protein immediately and become committed to meiosis. This may account for the tight co-regulation of conjugation and meiosis in *S.pombe*. In *mat1-Pm* mutants the cascade of *pat1* inactivation is blocked before meiosis and, in consequence, a 100-fold greater fraction of the zygotes are able to resume diploid mitosis when transferred to fresh growth medium (Egel, 1973a).

Our model can also explain induction of meiosis in *M/P* diploid cell lines upon nitrogen starvation. The major difference is that *mei3* is expressed directly after induction of the *mat1* transcripts because the *mat1-Pm* and *mat1-Mm* gene products are synthesized in the same cell. In haploid *P* cells the *M* pheromone signal is required for transcription of the *mat1-Pm* gene (Figure 1b), and further evidence suggests that a pheromone signal is also required for induction of meiosis in diploid cells. The meiotic block of *map1* and *mat1-Pc* mutant diploid strains can be rescued by a substance produced by wild-type *P* cells (Egel, 1973b; Meade and Gutz, 1976). Recently, this '*map1* substance' was shown to work over a distance in a manner similar to the *P* factor pheromone secreted by wild-type *P* cells (Leupold *et al.*, 1989a). We presume that this substance is identical to the *P* pheromone and that it allows meiosis by inducing transcription of the *mat1-Pm* gene. This suggests that the *mat1-Pm* gene can be transcribed in response to either the *P* or the *M* factor, which would be analogous to the situation in *S.cerevisiae* where the signal triggered by pheromone–receptor interaction is identical in the two mating types (Herskowitz and Marsh, 1987). The *P*-sterile *map1* and *mat1-Pc* mutants only possess functional *M*-receptors, so external provision of the *P*-phermone is required in order to generate a signal. We presume that wild-type diploid cells also require the pheromone signal in order to initiate meiosis, and that such cells synthesize the pheromones themselves.

In temperature-sentitive *pat1*-114 mutants growing on rich medium, the two first control points are derepressed at 30°C whereas the third control point is relieved at 35°C. However, inactivation of the wild-type *pat1* protein during differentiation is likely to be composed of three separate events, since three different levels of induction can be discerned in $pat1^+$ strains by genetic criteria. The $p52^{pat1}$ protein product is an auto-phosphorylating kinase with several phosphate groups attached when fully active (McLeod and Beach, 1988). One attractive hypothesis is that the various steps of the inactivation are paralleled by a change in kinase activity resulting in stepwise dephosphorylation of different subsets of substrates. Such alterations in substrate specificity could be accomplished by interactions with different proteins or may reflect a declining series of quantative thresholds in kinase activity. Full inactivation of $p52^{pat1}$ by the $p21^{mei3}$ protein *in vitro* completely abolishes autophosphorylation (McLeod and Beach, 1988).

### Nutritional signalling

The temperature-sensitive *pat1*-114 mutant ceases to grow at 35°C and undergoes untimely meiosis and sporulation. This growth deficiency can be suppressed by both physiological and genetic means, which appear to activate a *pat1*-independent repression of the differentiation process. Elevation of intracellular cyclic AMP concentration suppresses the ts sporulation phenotype of *pat1* mutants (Beach *et al.*, 1985), apparently because such conditions prevent transcription of the *mei2* gene (Watanabe *et al.*, 1988). Transcriptional induction of the *mat1* genes is also

prevented by cyclic AMP (cAMP). It is not clear, however, to what extent this nucleotide contributes to the normal signal that triggers conjugation and sporulation, since the intracellular level of cAMP seems not to fall substantially during cell differentiation (Beach *et al.*, 1985; Fukui *et al.*, 1986). It has been suggested that cAMP suppresses the ts sporulation phenotype of *pat1* mutants by hyperactivating a cAMP dependent protein kinase which shares substrates with $p52^{pat1}$ (McLeod and Beach, 1986). Our observations that the *pat1* kinase represses transcription of the *mat1* genes are compatible with that idea. Furthermore, the transcriptional induction of the *mat1-P* genes caused by inactivation of *pat1*-114 is delayed when cAMP is added (O.Nielsen, unpublished data). This suggests that the intracellular concentration of this nucleotide has to be reduced below a certain threshold before the cells start to differentiate.

In addition to *mei2* mutations, two other genetic conditions that suppress *pat1* have been described (Watanabe *et al.*, 1988): mtuations in a gene *steX* (which may be allelic to *aff1* defined by Sipiczki, 1988) and overproduction of a gene named *pac1* confer complete sterility upon the cells. These genetic changes also prevent transcription of the *mei2* gene and apparently mimic addition of cAMP.

In another group of sterile mutants, only the early steps of the differentiation process seem to be affected and not the *mei2* gene directly. Seven different genes have been identified (*ste1–ste4* and *ste7–ste9*), mutations of which prevent both conjugation and meiosis (Girgsdies, 1982; Michael and Gutz, 1987). When *pat1* activity is completely abolished in these strains, they all undergo meiosis, suggesting that these *ste* mutants still allow activation of the *mei2* gene. However, when *pat1* function is only partially impaired, two different mutant classes can be discerned (Sipiczki, 1988). Whereas *ste1*, *ste3* and *ste8* mutants conjugate (class IIa), *ste2*, *ste4*, *ste7* and *ste9* strains fail to do so (class IIb). Thus, the class IIb *ste* genes function downstream of *pat1* in conjugation and upstream of *pat1* in meiosis. This suggests that these *ste* gene products mediate the effect of *pat1* inactivation on induction of conjugation. According to this interpretation, the meiotic defect should be indirectly caused by inability to induce the *mat* genes and *mei3*. Mutants in two other genes (class I: *ste5/ras1* and *ste6*) are still able to undergo meiosis when diploid (*ras1* at very reduced frequency), and presumably play a more specific role in conjugation.

The class IIa *ste* genes, on the other hand, act very early in the differentiation process, upstream of the point where *pat1* represses conjugation. This suggests that the gene products of *ste1*, *ste3* and *ste8* may transform the signal of nutritional deprivation into an initial *pat1* inactivation event. The following observations support the notion that nitrogen starvation brings about differentiation through interaction with *pat1* (Leupold *et al.*, 1989b). Under conditions of nitrogen limitation *pat1*-114 driven meiosis occurs at a significantly lower temperature than in rich medium. Furthermore, when *pat1*-114 strains are starved for nitrogen in permissive conditions where meiosis still depends on mating types, the pheromone communication is stimulated compared to $pat1^+$ strains. However, the level of *pat1* activity may represent only one input among others affecting whether a cell decides to differentiate. Another such parameter could be the level of cAMP.

In this report we have studied a number of intermediate *pat1* effects—well before this protein kinase is completely inactivated and meiosis takes over. We have shown that the conjugation-related transcription pattern of the *mat1-P* genes can be reproduced by intermediate temperature shifts in a *pat1-ts* mutant, and we have proposed a regulatory model with multiple roles of *pat1*. Apparently, dynamic changes in *pat1* kinase activity orchestrate the sexual differentiation process in fission yeast. Unravelling the causes and consequences of the chain of *pat1* inactivation at the molecular level will provide interesting insights into the control of cell differentiation.

## Materials and methods

### *General S.pombe procedures*

Standard genetic techniques for *S.pombe* were as described by Gutz *et al.* (1974). PM and PM−nitrogen medium used for growing *S.pombe* cells in physiological experiments were prepared as described by Beach *et al.* (1985). Determination of the ability of cells to undergo conjugation and meiosis after temperature shift in *pat1*-114 mutant strains was according to Sipiczki (1988).

### *Physiological experiments*

The effect of nitrogen starvation on *mat1* RNA synthesis in $pat1^+$ strains was examined in the following way. Cells were grown in PM medium at 30°C to a density of $5 \times 10^6$ cells/ml. Half of the culture was used for RNA preparation and the other half was transferred to PM−nitrogen medium and grown at 30°C for 6 h before RNA preparation.

To monitor the effects of temperature shift-up in *pat1*-114 mutant strains, cells were grown in PM medium at 23°C to a density of $5 \times 10^6$ cells/ml. Half of the culture was used for RNA preparation while the other half was shifted to 30°C for 6 h before RNA preparation. In PM medium cells can be grown to stationary phase without induction of mating and sporulation because the nitrogen source is not depleted (Beach *et al.*, 1985; O.Nielsen, unpublished data).

### *Isolation of RNA*

The RNA preparation procedure described for budding yeast by Sherman *et al.* (1986) was modified for *S.pombe* in the following way. Twenty millilitres of cells were rapidly cooled to 0°C by mixing with an equal volume of crushed ice in a Sorvall SS34 Oak-Ridge tube. From this point all manipulations were performed at 0°C. Cells were harvested by centrifugation at 6000 *g* for 5 min, and resuspended in 0.5 ml LETS buffer (Sherman *et al.*, 1986). The cells were transferred to a 15 ml Corex tube containing 2.2 g glass beads (1 mm diameter) and 0.6 ml phenol equilibrated with LETS buffer. Cell breakage was accomplished by vortexing for a total of 3 min, in 30-s bursts alternating with intervals of 30-s incubation on ice. Then 1 ml of LETS buffer was added and the phases were separated by centrifugation at 8000 *g* for 5 min. Two 0.7 ml aliquots were collected from the supernatant into Eppendorf tubes. After extraction with phenol/chloroform (twice) and chloroform (once), the RNA was precipitated by 0.5 M LiCi, and resuspended in 100 μl $H_2O$. By this procedure the yield from a culture containing $5 \times 10^6$ cells/ml is 100–200 μg of total RNA.

### *Hybridization probes*

Substrates for *in vitro* transcription of strand-specific hybridization probes were constructed by cloning the relevant fragments into *pGEM3* (Promega Biotec). For detection of *P*-cell transcripts, a 904 bp *Hin*PI–*Mlu*I DNA fragment from *mat1-P* was used. This was transcribed into single-stranded RNA probes of either top-strand polarity (for detection of *mat1-Pc*) or bottom-strand polarity (*mat1-Pm*). These two genes are divergently transcribed. The hybridization probe used for detection of the *mat1-Mc* transcript was a 1016 bp *Bcl*I–*Taq*I DNA fragment from *mat1-M* transcribed into an RNA probe of appropriate polarity. The *in vitro* transcription reactions were carried out as described by Nielsen and Egel (1989).

### *Northern analysis*

Ten micrograms of total RNA from each of the experimental strains to be examined were run in parallel lanes on 1.5% formaldehyde gels and blotted on to Gene Screen membranes (NEN) according to the manufacturer's instructions. Northern hybridization to single-stranded RNA probes was performed as described by Sørensen *et al.* (1989).

## Acknowledgements

We thank Masayuki Yamamoto (via Paul Nurse) for providing the *pat1* strain and Herbert Gutz for the *mat1-Pc* strain. The expert technical assistance of Tove Friis is gratefully acknowledged. We also thank Dietmar Weilguny, Martin Willer and Unnur Styrkarsdottir for help and suggestions. We are grateful to Amar Klar for comments on the manuscript. This work was supported by the Danish Center for Microbiology.

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*Received on December 12, 1989; revised on February 12, 1990*