JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6325–6334 Vol. 179, No. 20
0021-9193/97/$04.00+0

Rapamycin Specifically Interferes with the Developmental Response of Fission Yeast to Starvation

R. WEISMAN,[1]* M. CHODER,[1] AND Y. KOLTIN[2]

Department of Molecular Microbiology and Biotechnology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel,[1] and Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts 02139[2]

Rapamycin is a microbial macrolide which belongs to a family of immunosuppressive drugs that suppress the immune system by blocking stages of signal transduction in T lymphocytes. In *Saccharomyces cerevisiae* cells, as in T lymphocytes, rapamycin inhibits growth and cells become arrested at the G_1 stage of the cell cycle. Rapamycin is also an effective antifungal agent, affecting the growth of yeast and filamentous fungi. Unexpectedly, we observed that rapamycin has no apparent effect on the vegetative growth of *Schizosaccharomyces pombe*. Instead, the drug becomes effective only when cells experience starvation. Under such conditions, homothallic wild-type cells will normally mate and undergo sporulation. In the presence of rapamycin, this sexual development process is strongly inhibited and cells adopt an alternative physiological option and enter stationary phase. Rapamycin strongly inhibits sexual development of haploid cells prior to the stage of sexual conjugation. In contrast, the drug has only a slight inhibitory effect on the sporulation of diploid cells. A genetic approach was applied to identify the signal transduction pathway that is inhibited by rapamycin. The results indicate that either rapamycin did not suppress the derepression of sexual development of strains in which adenylate cyclase was deleted or the cyclic AMP-dependent protein kinase encoded by *pka1* was mutated. Nor did rapamycin inhibit the unscheduled meiosis observed in *pat1-114* mutants. Overexpression of $ras1^+$, an essential gene for sexual development, did not rescue the sterility of rapamycin-treated cells. However, expression of the activated allele, $ras1^{Val17}$, antagonized the effect of rapamycin and restored the ability of the cells to respond to mating signals in the presence of the drug. We discuss possible mechanisms for the inhibitory effect of rapamycin on sexual development in *S. pombe*.

Rapamycin, cyclosporin A (CsA), and FK506 are microbial products which exhibit immunosuppressive activity (1, 5, 6, 21, 41, and 70). The importance of the immunosuppressive drugs as therapeutic agents has catalyzed a search to define their molecular mechanism of action. Studies of this mechanism have been carried out mainly with certain types of mammalian cells and with the budding yeast *Saccharomyces cerevisiae*. These studies have suggested that several features of the mode of action of the drugs are conserved throughout evolution. In all systems studied, it was found that the three immunosuppressants bind with high affinity to cytoplasmic receptors termed immunophilins (reviewed in references 28 and 69). CsA binds cyclophilin, whereas FK506 and rapamycin, which are structurally related, bind a different immunophilin called FKBP. Both cyclophilins and FKBPs exhibit similar enzymatic activities of peptidyl-prolyl *cis-trans* isomerization.

Complexes CsA-cyclophilin and FK506-FKBP inhibit the T-cell receptor signalling pathway by binding to and inhibiting the activity of the Ca^{2+}-dependent protein phosphatase, calcineurin. As a consequence, the translocation from the cytoplasm to the nucleus of the T-cell transcription factor, NF-AT, is inhibited (15, 51). In *S. cerevisiae*, CsA-cyclophilin and FK506-FKBP complexes block the recovery from G_1 arrest induced by the mating pheromone (25) and inhibit vegetative growth in most strains (40, 74). The physiological target of the drug-immunophilin complexes is the yeast calcineurin (12, 25). In fission yeast *Schizosaccharomyces pombe* CsA causes an abnormal multiseptated phenotype (76, 80). This abnormal phenotype is assumed to result from the inhibition of calcineurin activity (80).

The rapamycin-FKBP complex does not affect calcineurin activity but binds the *S. cerevisiae* proteins encoded by the TORs. The human homologs are known as FRAP, RAFT, RAPT1, and mTOR (7, 13, 66, 67). The TORs and their mammalian counterparts are large proteins that contain a phosphatidylinositol 3-kinase motif. It is noteworthy that the human FRAP protein is a rapamycin-sensitive regulator of mitogen-stimulated p70 S6 kinase ($p70^{S6k}$) (8). The activity of $p70^{S6k}$ itself has been previously reported by numerous other studies to be inhibited by rapamycin (11, 14, 23, 43, 64). However, it is not yet clear which are the main downstream effectors of the rapamycin-sensitive pathway. Several such proteins, whose activity is either dependent on or independent of $p70^{S6k}$ activity, have been suggested. One line of investigation suggests that the main downstream effectors are involved in the control of protein synthesis, control that results from the inhibition of the phosphorylation of ribosomal protein S6 by $p70^{S6k}$ (14, 36, 73). Rapamycin may also inhibit the process of cap-dependent translation initiation by inhibition of the phosphorylation of the eIF-4E binding protein (4, 9, 47). Other suggestions are that the downstream effectors of the rapamycin-sensitive pathway are involved in transcriptional regulation. For example, rapamycin inhibits the phosphorylation of transcription factor CREMτ. As a result, transcriptional activation of genes, which normally occurs following stimulation of the adenylyl cyclase pathway, is inhibited (17). CREMτ is a member of the cyclic AMP (cAMP)-responsive element-binding factors. The possibility that these factors may play a role in the rapamycin-sensitive pathway is further supported by the finding that rapamycin inhibits the transcription of proliferating cell nuclear antigen by preventing the binding of cAMP-responsive element-binding elements to the promoter (24).

Rapamycin also inhibits the interleukin 2-induced activity of cyclin-dependent kinases (CDKs) Cdc2 and Cdk2 (2, 56, 60). This inhibition is thought to result from the inhibition of the degradation of CDK inhibitor Kip1 (60). The molecular mech-

* Corresponding author. Fax: 972-3-6409407. E-mail: ronitt@post.tau.ac.il.

anism that links the inhibition by rapamycin of TOR proteins and CDKs is yet to be elucidated (for a review, see reference 1).

Studies of the effect of rapamycin on *S. cerevisiae* have led to a better understanding of the cellular response to rapamycin at the molecular level. Rapamycin causes cell growth arrest in *S. cerevisiae*, and cells arrest early in the G_1 phase of the cell cycle (3, 31, 39). As in mammalian cells, the toxic effect of the drug requires the formation of a complex with the yeast FKBP (31, 39). Two related phosphatidylinositol 3-kinase-like proteins, TOR1 and TOR2, that were identified by genetic screens, are the targets of the rapamycin-FKBP12 complex (10, 31, 32, 42). The loss of TOR function results in the inhibition of translation initiation and induces several other physiological changes characteristic of starved cells entering stationary phase (3). These results suggest that the TOR signalling pathway regulates translation initiation in response to nutrient growth signals (3, 19).

Thus far, the effect of rapamycin on *S. pombe* has not been reported. *S. pombe* is a well-studied yeast that is widely used as a model organism for higher eukaryotes, especially in studies on cell cycle regulation (48, 62). Concerning its evolution, it is distantly related to the budding yeast *S. cerevisiae* and in some respects is more similar to higher eukaryotes than to *S. cerevisiae* (65, 71).

S. pombe multiplies primarily in the haploid state. Haploid cells have two mating types, h^+ (P) and h^- (M) (reviewed in reference 22). Wild-type *S. pombe* cells are homothallic (h^{90}); that is, they can switch their mating types between h^+ and h^- every other generation. Heterothallic strains, which express either h^+ or h^- mating type phenotypes, are also available. Upon starvation cells can become committed either to sexual differentiation or to an advance into stationary phase. If the sexual differentiation pathway is chosen, cells of opposite mating types conjugate to form diploid zygotes. If starvation conditions prevail, the diploid zygotes undergo meiosis and sporulation (reviewed in reference 22).

cAMP plays a critical role in the regulation of sexual development in *S. pombe*. Addition of cAMP to the medium or mutants which produce a high intracellular cAMP level inhibit sexual differentiation, whereas attenuation of the cAMP pathway results in a propensity to sporulate even in rich nutrient growth conditions (49, 79). Entry into the sexual pathway is also dependent on the establishment of the mating pheromone signalling pathway. The single *S. pombe* homolog of the mammalian Ras oncoprotein, the *ras1*$^+$ gene product, is essential for sexual differentiation and is thought to modulate sensitivity to the mating pheromones (26, 58, 59; reviewed in reference 33). Remarkably, the *S. pombe ras1*$^+$ gene product is not essential for vegetative growth (26, 58). As in mammals, the *ras1*$^+$ product participates in the regulation of a protein kinase cascade that ultimately regulates a member of the mitogen-activated protein kinase family. In *S. pombe* this cascade is composed of the products of *byr2*$^+$, *byr1*$^+$, and *spk1*$^+$ (29, 57, 75).

We report that rapamycin does not affect vegetative growth and entrance into stationary phase in *S. pombe* but has a distinct effect on sexual development at an early stage prior to conjugation.

MATERIALS AND METHODS

Yeast strains, media, and general techniques. The *S. pombe* strains used for this study are listed in Table 1. All media used in this study are based on those described previously (55) and are described in more detail in reference 72. Edinburgh minimal medium (EMM)-N contains no nitrogen; EMM lowG contains 0.1% glucose. Cells were grown and experiments were done at 25°C, unless otherwise indicated.

TABLE 1. Strains used in this study

Strain	Description
ED812[a]	972 h^-
ED787[a]	975 h^+
ED666[a]	*ura4-D18 leu1-32 ade6-M210* h^+
ED667[a]	*ura4-D18 leu1-32 ade6-M216* h^+
TA07[d]	*ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210* h^+/h^-
TA22[d]	*leu1-32* h^+
TA23[d]	*leu1-32* h^-
ED1091[a]	*pat1-114* h^-
ED1054[a]	*pat1-114 leu1-32* h^-
ED813[a]	h^{90}
SP837[b]	*ura4-D18 leu1-32 ade6-M216* h^{90}
CHP364[c]	*pka1-216 leu1-32 ade6-M216* h^{90}
CHP559[c]	*git2-1::LEU2 Fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216* h^{90}

[a] P. Fantes and S. MacNeill, Edinburgh University, Edinburgh, United Kingdom.
[b] A. Cohen, Hebrew University, Jerusalem, Israel.
[c] C. S. Hoffman, Boston College, Chestnut Hill, Mass.
[d] This work.

Rapamycin was purchased from Biomol Research Laboratories, Plymouth, Mass. For the stock solution, 1 mg of rapamycin was dissolved in 1 ml of 1:1 dimethyl sulfoxide (DMSO)-methanol solution and kept at −20°C. Rapamycin was added to a final concentration of 0.2 μg/ml in liquid- or agar-containing media, unless otherwise indicated. An equal volume of the drug vehicle solution (1:1 DMSO-methanol) was used as a control in all experiments.

Standard genetic procedures were performed as described in references 30 and 38. Transformation of *S. pombe* cells was performed by electroporation (63). General molecular techniques were performed as described in reference 68.

Assays for mating efficiency. Cells were grown at 25°C in EMM, appropriately supplemented with amino acids when required, to a density of approximately 5×10^6 cells/ml. Then, the cultures were washed three times with double-distilled water and reinoculated into nitrogen-free liquid medium (EMM-N) at a density of approximately 5×10^6 cells/ml and further incubated at 25°C. Aliquots were taken at the indicated time points, and after gentle sonication, the numbers of cells, zygotes, and spores were counted under the microscope. Whenever solid EMM-N medium was used, 5 μl of medium containing 5×10^6 cells was spotted. After 3 days of incubation at 25°C, a toothpick was used to pick some of the cells from the center of each patch, and the cells were briefly sonicated and examined microscopically.

The percentage of mating was calculated by dividing the number of zygotes, asci, and free spores by the number of total cells. One zygote or one ascus was counted as two cells, and one spore was counted as a half cell. In each experiment 500 to 1,000 cells were counted.

Assay for sporulation efficiency of diploids. A diploid strain (TA07) was constructed according the method described by Moreno et al. (55). Diploid cells were grown and starved as described for haploid cells in the mating assay. After 3 days of incubation in starvation medium, aliquots were taken and the cells were briefly sonicated and examined microscopically. The percentage of sporulation was calculated by dividing the number of asci and free spores by the number of total cells. One ascus was counted as two cells, and one spore was counted as a half cell. In each experiment 500 to 1,000 cells were counted.

Determination of cell viability during stationary phase and after heat shock. Cells were grown in EMM at 25°C to mid-log phase in the presence or absence of rapamycin. The cells were then washed and reinoculated into EMM-N in the presence or absence of rapamycin. Aliquots were sampled at the indicated times. Cells were counted, appropriately diluted, and plated onto yeast extract (YE) plates. Cell viability was determined by the capacity of cells to form colonies on supplemented YE at 30°C.

Cells were exposed to heat shock after 5 days of incubation in EMM-N. Aliquots were taken and incubated at 47°C for various times. Viability was determined as described above.

RESULTS

Rapamycin does not inhibit vegetative growth in *S. pombe*. Rapamycin shows a strong inhibitory effect on vegetative growth of *S. cerevisiae* cells at a concentration of <0.1 μg/ml (31, 39). Surprisingly, we found that rapamycin did not inhibit

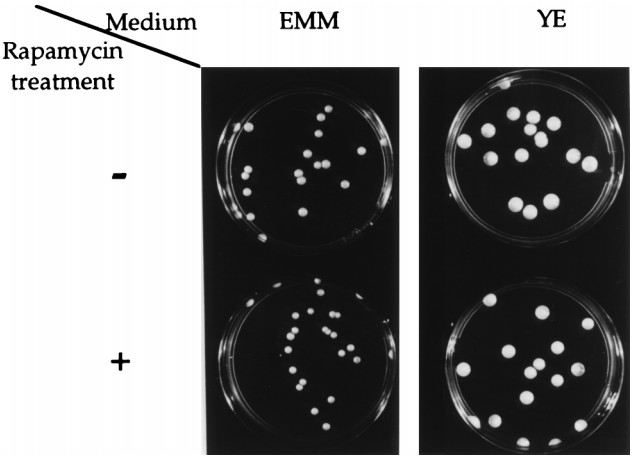

FIG. 1. Rapamycin does not affect wild-type h^+ colony growth. Cells were plated on EMM or YE plates containing rapamycin or the drug vehicle alone. Colonies are larger on rich (YE) plates than on minimal (EMM) plates, but the size is not affected by the presence of rapamycin.

the growth of *S. pombe*. Similar-size colonies were obtained when wild-type homothallic (h^{90}) or heterothallic (h^+ or h^-) strains were grown on either complex or minimal medium in the presence (0.2 or 0.5 μg/ml) or absence of rapamycin. This insensitivity to the drug is shown also by cells grown at a range of temperatures between 20 and 35°C (Fig. 1 and data not shown). A microscopic examination did not reveal any distinguishable morphological changes associated with the rapamycin treatment, and cells divided at the same cell size as untreated cells (see Fig. 2A and B). The doubling time of the wild-type h^+ strain in liquid complex medium at 32°C in the presence of rapamycin was similar to that of the control without the drug (135 and 125 min, respectively). These results indicate that, in contrast to its effect on the vegetative growth of *S. cerevisiae*, rapamycin does not interfere with the vegetative growth of *S. pombe.*

Rapamycin strongly inhibits conjugation in *S. pombe.* In a series of experiments we examined the ability of rapamycin-treated cells to respond to conditions which normally induce opposite-mating-type cells to undergo sexual differentiation. These conditions primarily consist of nutrient deprivation (reviewed in reference 22). Conventionally, either a low-nutrient medium such as malt extract (ME), a low-nitrogen medium (EMM-N), or a low-glucose medium (EMM lowG) is used for strong induction of sexual differentiation. Table 2 depicts the mating efficiencies which are obtained when opposite-mating-type cells are starved in the presence of 0.2 μg of rapamycin per ml. The results indicate that under any of the starvation conditions used, the mating efficiency is strongly reduced (9- to 60-fold) in the presence of the drug.

Mating requires that the opposite mating types, h^+ and h^-, be present in close proximity. These conditions can be achieved either in homothallic strains, which switch their mating types between h^+ and h^-, or in a mixture of opposite mating types of heterothallic strains. When a homothallic strain or heterothallic strains were starved in the presence of rapamycin, no significant difference between the inhibitory effects of rapamycin on the homothallic strain and on the mixture of heterothallic strains was observed. These results indicate that rapamycin does not interfere with the process responsible for mating type switching in homothallic strains.

Figure 3 demonstrates the inhibitory effect of rapamycin on the entry into the sexual differentiation pathway of wild-type homothallic h^{90} cells. Upon a shift to EMM-N in the absence of the drug, cells gradually conjugated until a final value of 48% was reached. Cells that were starved in the presence of rapamycin reached a maximum mating efficiency of only 7%. Microscopic examination revealed that the morphology of the starved cells in the presence of rapamycin was very similar to that of stationary (resting) haploid cells. In the presence of rapamycin cells arrested at a small cell size, typical for starved cells, and did not develop conjugation tubes (Fig. 2; compare panels C and D). In addition, an effect on agglutination was observed. Cells of the untreated culture started to agglutinate within 23 h, whereas the rapamycin-treated cultures did not agglutinate during the course of the experiment (Fig. 2; compare panels C and D). Sexual agglutination is an early indication of sexual activity (reviewed in reference 22). The lack of agglutination in the presence of rapamycin suggests that rapamycin inhibits one of the early sexual differentiation stages.

Vegetative growth in the presence of rapamycin causes mating incompetence. Although rapamycin has no apparent effect on the vegetative growth of *S. pombe*, we examined the possibility that rapamycin affects growing cells but that the effect is only observed upon starvation. To this end, homothallic cells were grown to exponential phase in the presence of rapamycin. Cells were then washed three times and reinoculated into EMM-N in the absence of rapamycin. We next determined whether the cells underwent normal sexual differentiation once they were removed from the drug.

The exposure to rapamycin during the growth phase had a dramatic effect on the ability of the cells to undergo sexual differentiation. More than a 20-fold reduction in mating efficiency in EMM-N after exposure to the drug during growth was observed, compared to the efficiency observed with untreated cells (Fig. 3 and 4). A further decrease in mating efficiency was observed if cells were grown and then starved in the presence of rapamycin: a more than 500-fold reduction in mating efficiency in comparison with cells that were not exposed to rapamycin (Fig. 3 and Fig. 4). Thus, pregrowth with rapamycin rendered cells incompetent for mating. This suggests that the target of rapamycin exists during vegetative growth. One other explanation is that the drug blocks, in an irreversible way, a biochemical pathway, thereby not allowing cells to become committed to the sexual differentiation pathway.

Since growth in the presence of rapamycin affects subsequent sexual differentiation, we were able to examine whether rapamycin affects cells of both mating types equally or is mating type specific. We examined mating efficiency in crosses of wild-type heterothallic strains in which only one mating type was grown in the presence of rapamycin. We found that, irrespective of which of the mating types was grown with rapamycin, the mating efficiency was reduced 2.5-fold relative to that for crosses of untreated strains of opposite mating types. Thus, the function affected by rapamycin is one that needs to be active in both mates. When both mating types were grown in the presence of rapamycin and then plated on drug-free plates, the mating efficiency was reduced ninefold relative to that for crosses of untreated cells. Therefore, the mating efficiency observed in crosses in which only one mating type was grown in the presence of rapamycin is an intermediate one, indicating that untreated cells can partially suppress the effect of rapamycin.

The differences in the mating efficiencies of the above crosses can be demonstrated by exposing patches of the crosses to iodine vapor (Fig. 5). As deprivation of nutrients is also a signal for entry into meiosis, newly formed zygotes rapidly undergo meiosis and sporulation. Carbohydrate accumulation

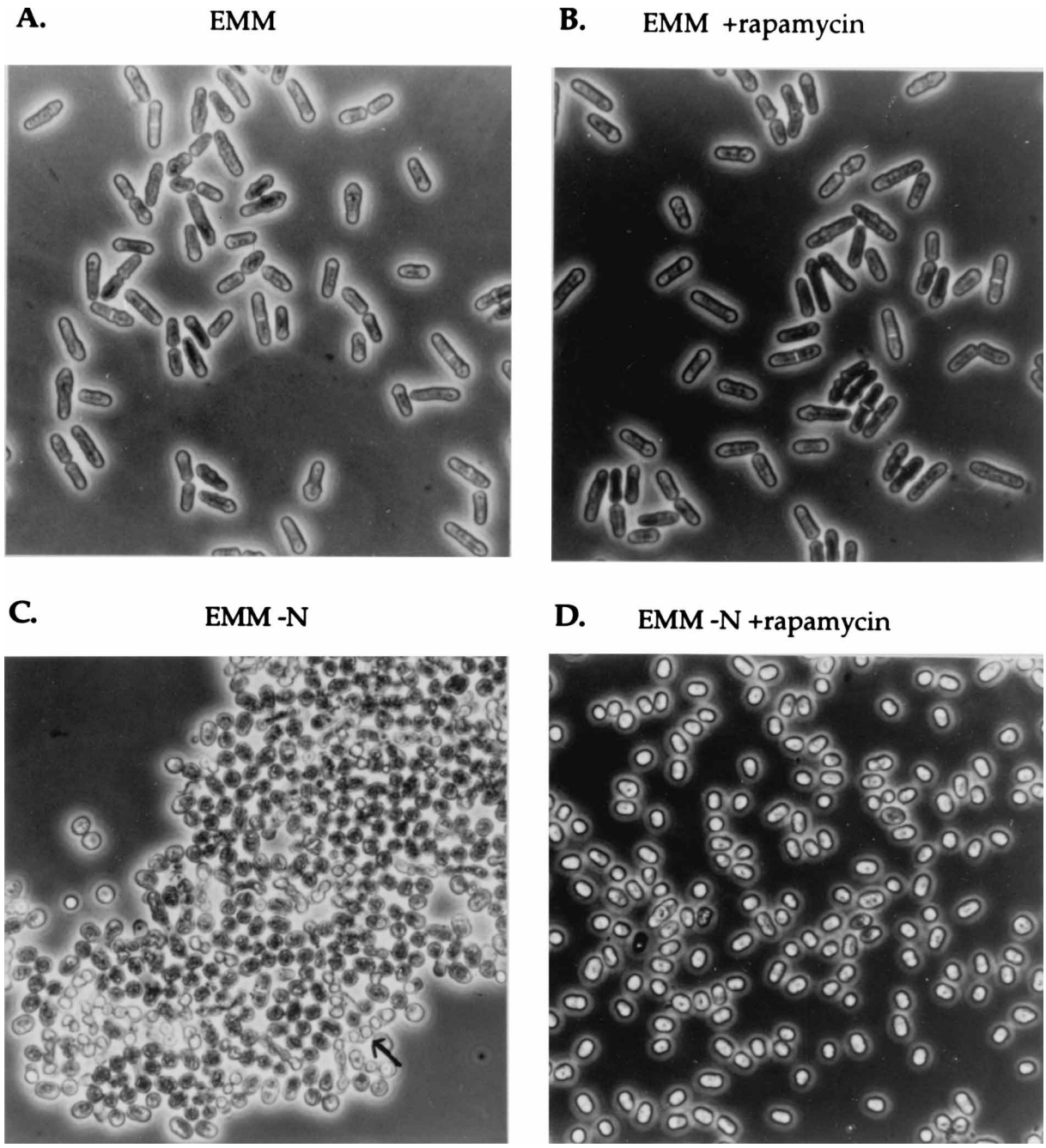

FIG. 2. Rapamycin does not affect the morphology of growing cells but exerts a dramatic effect on the morphology of nitrogen-starved cells. (A and B) h^{90} wild-type (ED813) cell samples were taken from an exponentially growing culture in EMM supplemented with the drug vehicle alone (A) or with rapamycin (B). (C and D) h^{90} wild-type (ED813) cell samples were taken 3 days after a shift from EMM to liquid EMM-N containing the drug vehicle (C) or rapamycin (D).

is a hallmark of the sporulation process and can be detected by staining with iodine vapor (45, 61). Figure 5 (left panel) demonstrates that the patch of a cross of untreated cells is stained dark, indicating the presence of spores. The patch of a cross in which both mating types were pretreated with rapamycin appears yellow, indicating the lack of sporulation. The patches of crosses in which only one mating type was pretreated with rapamycin appear greyish, indicating reduced sporulation.

Rapamycin has only little or no effect on the sporulation of diploids. To determine whether rapamycin affects only the early stages in sexual differentiation or whether it may affect some later stages, such as sporulation, we constructed a diploid strain by using two haploid strains with complementing alleles of the *ade6* gene (see reference 55). The diploid strain (TA07) was formed by crossing strain ED667 (*ura4-D18 leu1-32 ade6-M216* h^+) with ED666 (*ura4-D18 leu1-32 ade6-M210* h^-) and determining the adenine prototrophs among the progeny.

Because the diploid resulted from the mating of two auxotrophic mutants we first examined whether the nutritional markers had any effect on the sensitivity to rapamycin during conjugation. When ED666 was crossed with ED667 on EMM-N plates in the presence of rapamycin, a ninefold re-

TABLE 2. Inhibition of mating by treatment with rapamycin[a]

Medium composition	Mating type	Mating efficiency (%)
EMM-N	h^{90}	58
EMM-N + rapa	h^{90}	5
EMM lowG	h^{90}	52
EMM lowG + rapa	h^{90}	6
EMM-N	$h^+ \times h^-$	59
EMM-N + rapa	$h^+ \times h^-$	1
ME	$h^+ \times h^-$	60
ME + rapa	$h^+ \times h^-$	4

[a] h^{90} (ED813) cells and a 1:1 mixture of h^+ (ED787) and h^- (ED812) cells were grown in the absence of rapamycin until mid-log phase; cells were then plated on solid media with the indicated compositions in the presence or absence of rapamycin (rapa). Mating efficiency was calculated as described in Materials and Methods. The results are the mean values of two separate experiments.

duction in the mating efficiency compared with that for the same cross in the absence of rapamycin was observed (data not shown). Thus, the auxotrophic traits used in the cross have no effect on the drug sensitivity.

The effect of rapamycin on the sporulation of diploid TA07 was determined. Interestingly, no significant decrease in sporulation was observed when the diploid cells were induced to sporulate in the presence of rapamycin (Fig. 6; first and second bars from the left). A microscopic examination of diploid cells which were sporulated in the presence of rapamycin revealed that asci containing four spores were formed with the same efficiency as that in the absence of the drug. However, a small decrease in sporulation efficiency (2.4-fold) was observed when the diploid cells had been grown in the presence of rapamycin before subjecting them to nitrogen-limiting conditions (Fig. 6; third bar from the left). Note that the results of the latter experiment should be compared to those of similar experiments done with haploids, where the inhibitory effect when cells had been grown in the presence of rapamycin was more than 500-fold (Fig. 3 and 4). Therefore, it is concluded that, whereas rapamycin has a dramatic inhibitory effect on sexual development of starved haploids, it exerts little or no effect on the sporulation of starved diploids. This observation reinforces our conclusion that the drug effect is specific to an early stage of developmental response to starvation, before mating is even initiated, and has little or no effect on later stages.

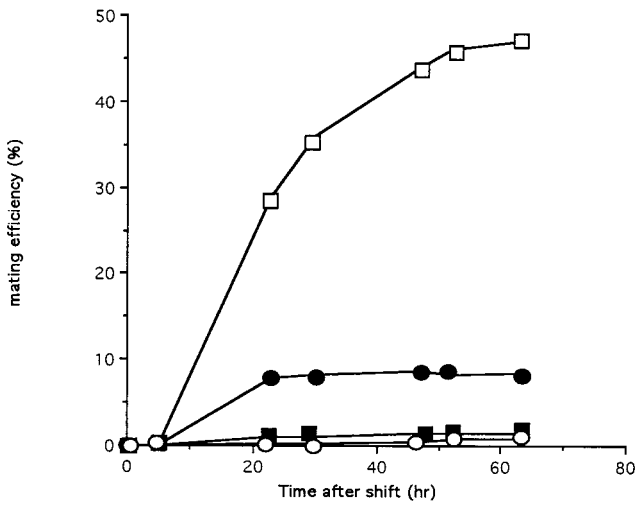

FIG. 3. Inhibition of mating by rapamycin. h^{90} wild-type cells (ED813) growing exponentially in EMM in the presence of the drug vehicle alone (□, ●) or rapamycin (■, ○) were washed three times with water and recultured at ~5 × 10^6 cells/ml in EMM-N medium containing the drug vehicle (□, ■) or rapamycin (●, ○). At the indicated time points, samples were removed and the mating efficiencies were determined as described in Materials and Methods.

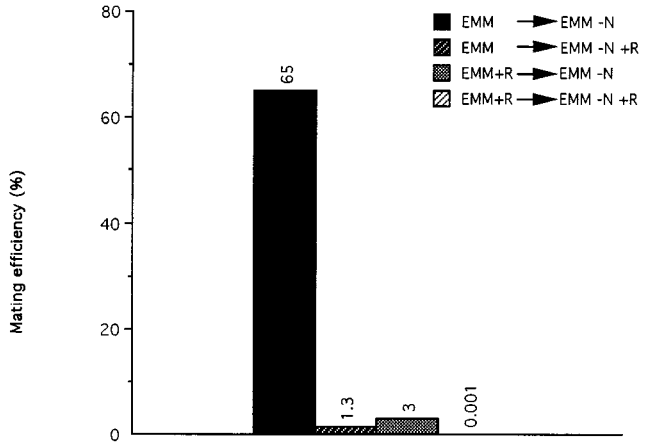

FIG. 4. Rapamycin inhibits mating of heterothallic strains. Heterothallic h^+ or h^- cells were grown in EMM in the presence of the drug vehicle alone (EMM) or in the presence of rapamycin (EMM+R). In mid-log phase, cells were harvested and washed, and the two opposite-mating-type cultures were mixed together at a 1:1 ratio. A total of 5 × 10^6 cells were plated on an EMM-N plate supplemented either with the drug vehicle (EMM-N) or with rapamycin (EMM-N+R). The mating efficiency was determined at 3 days postplating, as described in Materials and Methods.

Treatment with rapamycin does not interfere with entrance into stationary phase. The transition of *S. pombe* cells from the mitotic cycle to the meiotic sexual cycle occurs at the G_1 phase (reviewed in reference 22). Under nitrogen starvation conditions, cells become arrested in the G_1 phase and either enter the stationary phase, analogous to the mammalian G_0 state, or

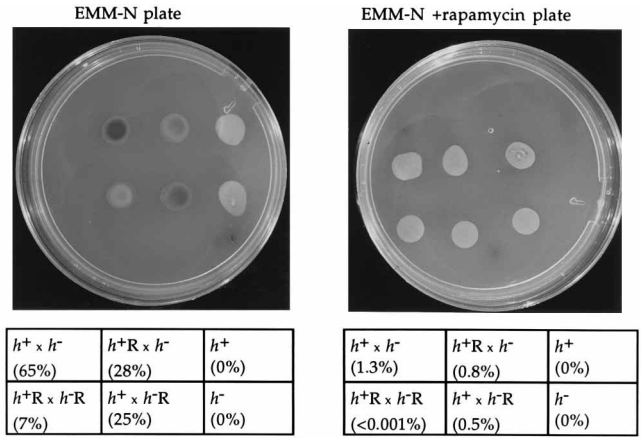

FIG. 5. Untreated cells partially suppress the sterility of rapamycin-treated cells. Heterothallic h^+ or h^- cells that were pregrown in the presence of either the drug vehicle alone or rapamycin were mixed together and plated on EMM-N plates with no rapamycin (left) or with rapamycin (right). Plates were incubated for 3 days and then stained with iodine vapor. None of the patches on the rapamycin-containing plate (right) is stained with dark color, as rapamycin inhibits sexual development. On the plate with no rapamycin (left) various degrees of dark staining are detected, depending on whether or not the partners of the crosses were pregrown in the presence of rapamycin. The table below the plates indicates, for each patch, the pretreatment applied for each partner of the cross. R indicates pregrowth with rapamycin. The numbers in parentheses indicate mating efficiencies as observed by microscopic examination.

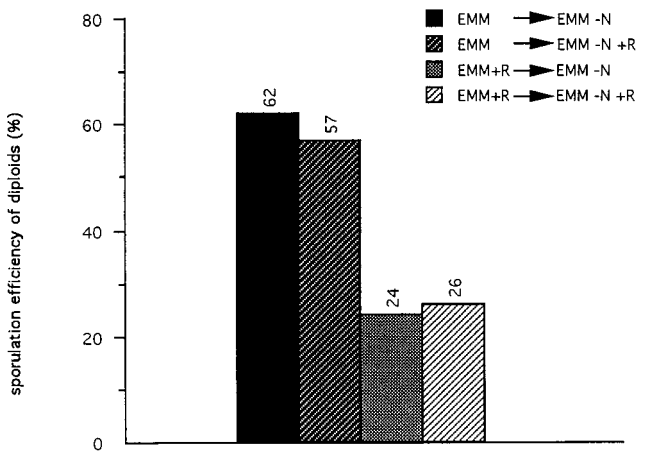

FIG. 6. Rapamycin has a small inhibitory effect on the sporulation of a diploid. TA07 diploid cells were grown in EMM in the presence of the drug vehicle alone (EMM) or rapamycin (EMM+R). In mid-log phase, cells were harvested, washed, and plated on EMM-N with the drug vehicle or rapamycin. The sporulation efficiencies were determined 3 days postplating.

initiate sexual differentiation. Some of the mutants which are impaired in sexual differentiation are also impaired in the capability of entering the stationary phase. Such mutants show low viability under starvation conditions (for examples see references 37 and 54).

In order to determine the effect of rapamycin on the ability of cells to enter the stationary phase, we examined the viability of cells which were starved in EMM-N in the presence of rapamycin. h^+ and h^{90} cells were grown in EMM to mid-log phase in the presence of rapamycin and then reinoculated into EMM-N in the presence of rapamycin. Rapamycin inhibited the mating activity of the h^{90} culture (resulting in less than 0.01% mating efficiency). We monitored the viability of the cells that (presumably) remained haploid, as demonstrated in Fig. 7. The results show that cell viability in long-term stationary-phase cultures in EMM-N was not significantly affected by

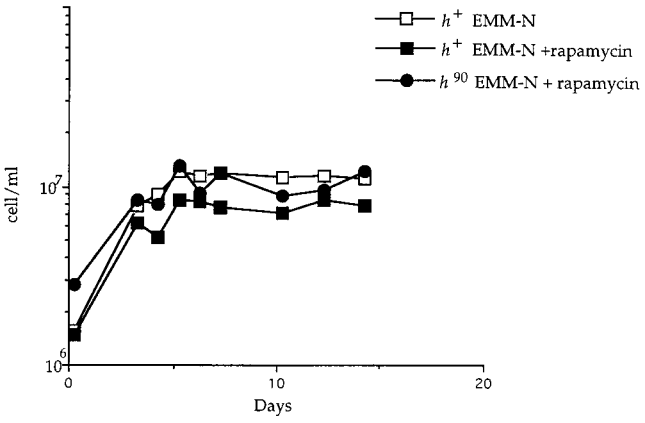

FIG. 7. Rapamycin does not affect cell viability in long-term stationary cultures. Wild-type h^+ and h^{90} cells were grown in EMM to mid-log phase in the absence or presence of rapamycin and then shifted into EMM-N in the absence or presence of rapamycin, respectively. After the shift to the nitrogen-limiting medium, samples of the cultures were aliquoted at the indicated times. Each sample was counted, appropriately diluted, and plated on YE plates. The number of colonies formed on each plate was counted, and the inferred concentration of viable cells at each time point was calculated.

TABLE 3. Effect of rapamycin treatment on the mating efficiencies of strains overexpressing *ras1*$^+$ and *ras1*^{Val17}[a]

Medium	Plasmid[b]	Mating efficiency (%)
EMM-N	pREP1 (vector)	63
EMM-N + rapa	pREP1 (vector)	15
EMM-N	pDB248′ras1	52
EMM-N + rapa	pDB248′ras1	8.0
EMM-N	pDB248′ras1-Val17	8.0
EMM-N + rapa	pDB248′ras1-Val17	<0.01

[a] SP837 h^{90} cells transformed with the indicated plasmids were grown to mid-log phase in appropriately supplemented EMM and then washed. A total of $\sim 5 \times 10^6$ cells were plated on appropriately supplemented EMM-N plates in the presence or absence of rapamycin (rapa). Mating efficiency was calculated as described in Materials and Methods.

[b] Plasmids pDB248′ras1 and pDB′ras1-Val17 were kindly provided by D. Hughes, Chester Beatty Laboratories, London, United Kingdom.

rapamycin. Note that upon the shift to the nitrogen limiting medium the untreated h^+ culture continued to divide several times before being completely arrested (Fig. 7). It is clear that the rapamycin-treated cultures also divided a few times before arrest, although the number of cell divisions before they were arrested is slightly smaller than that for the untreated culture.

The fact that rapamycin does not affect the entrance into stationary phase can be shown by other experimental procedures. One of the characteristics of *S. pombe* stationary cells is the acquisition of heat resistance (16). Cells in stationary phase can tolerate a heat shock of 47°C. Therefore, 5 days after incubation in EMM-N, the rapamycin-treated h^+ and h^{90} cultures (see above) were subjected to heat shock at 47°C, and cell survival values were compared with that of heat-shocked h^+ untreated cells. The results obtained indicated that cells of the different cultures were equally resistant to heat shock (data not shown). Thus, the above experiments indicate that rapamycin-treated cells enter and sustain the stationary phase normally.

An activated allele of *ras1*, *ras1*^{Val17}, restores the ability of rapamycin-treated cells to respond to mating signals. The phenotypic effect of rapamycin resembles the effect of the loss of function of several known genes that play a role in sexual differentiation. More than 20 genes required for mating have been identified. One of these genes is *ras1*$^+$, a close homolog of the mammalian *ras* proto-oncogene (26, 58). Haploid cells carrying a *ras1* null mutation are viable but cannot mate or respond to mating pheromones. In contrast, cells carrying the constitutively activated allele, *ras1*^{Val17}, are hyperresponsive to the pheromones. Such cells grow normally under vegetative conditions, but upon starvation and in the presence of both mating types they produce long conjugation tubes and mate poorly (27, 34). Taken together, these results suggest that a transient pulse of *ras1* activity is required early after nitrogen starvation (reviewed in reference 22).

We examined the effect of the overexpression of the wild-type *ras1*$^+$ and activated *ras1* alleles on rapamycin-treated cells. Overexpression of *ras1*$^+$ did not have any effect on cells that were grown or starved in the presence of rapamycin, and no suppression of the defect in conjugation was observed (Table 3). However, overexpression of *ras1*^{Val17} seemed to restore the ability of cells to respond to mating pheromones. Under starvation conditions cells produced elongated conjugation tubes, even in the presence of rapamycin (Fig. 8). This result suggests that rapamycin may affect a very early step of the mating pathway, upstream of the step determined by *ras1*. It is yet to be determined whether the targets of rapamycin and the *ras1*$^+$ protein lie on the same pathway. Remarkably, rapamycin

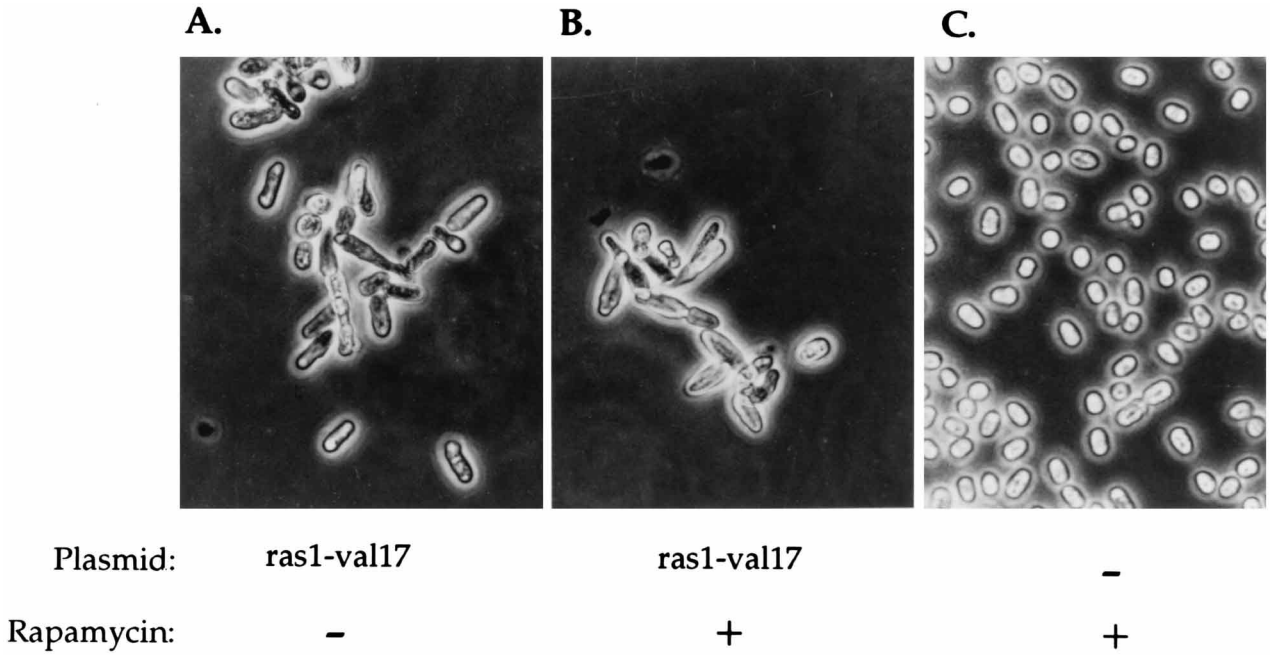

FIG. 8. Rapamycin does not inhibit agglutination and development of conjugation tubes of cells carrying the *ras1*^{Val17} allele. SP837 cells carrying pDB248′ras1-Val17 were grown in EMM to mid-log phase, then washed three times with water and recultured in EMM-N in the presence of the drug vehicle (A) or of rapamycin (B). Cells lacking the plasmid and treated with rapamycin are shown in panel C. Elongation of conjugation tubes and agglutination, both in the presence and in the absence of rapamycin, can be seen in cells expressing *ras1*^{Val17} but not in the parental wild-type cells.

does not seem to exert its effect via the Ras1 pathway in mammalian cells (53). Thus, whether or not rapamycin affects directly the *S. pombe ras1*$^+$ pathway remains an intriguing open question.

Rapamycin does not inhibit the phenotypes associated with mutants of the cAMP/pka1-dependent pathway. The *pat1*$^+$ gene (previously referred to as *ran1*$^+$) encodes a protein kinase which represses sexual differentiation and which is essential for the maintenance of vegetative cell division and growth (35, 52, 61). Loss of function of *pat1*$^+$ causes unscheduled meiosis, irrespective of ploidy, mating type, and growth conditions. The *pat1-114* strain is a temperature-sensitive mutant with a distinctive phenotype at the restrictive temperature, i.e., growth cessation and the development of irregular numbers of spores. Tests conducted with this mutant indicate that rapamycin does not suppress the uncontrolled meiosis of *pat1-114* cells at the restrictive temperature (Fig. 9). These results suggest that rapamycin is not involved in the suppression of functions that are required for the expression of the *pat1-114* lethal phenotype. Conditions that are able to suppress *pat1*-driven meiosis and the associated growth defect include an increase in the level of intracellular cAMP and overexpression of *pka1*$^+$, the gene for the cAMP-dependent kinase (49). Therefore, the inability of rapamycin to suppress the *pat1-114* lethal phenotype also suggests that rapamycin is not involved in the stimulation of the cAMP/pka1 pathway. In agreement with this suggestion are the observations that rapamycin does not interfere with aspects of vegetative growth or meiosis (data presented above). Such aspects of the life cycle of *S. pombe* can be affected by alternations in intracellular cAMP levels (reviewed in reference 79).

In addition, we found that rapamycin did not suppress mutants of the cAMP/pka1 pathway which are derepressed for sexual development. Mutants defective in the genes coding for adenylate cyclase (*git2*, also known as *cyr1*), which converts ATP into cAMP, or the catalytic subunit of protein kinase A (*pka1*, also known as *git6*) readily mate even in rich medium. We found that the mating efficiency of either *git2* or *git6* mutants in either complete or minimal medium was not affected by rapamycin (data not shown). Therefore, it is evident that rapamycin does not exert its effect downstream of the cAMP-dependent protein kinase.

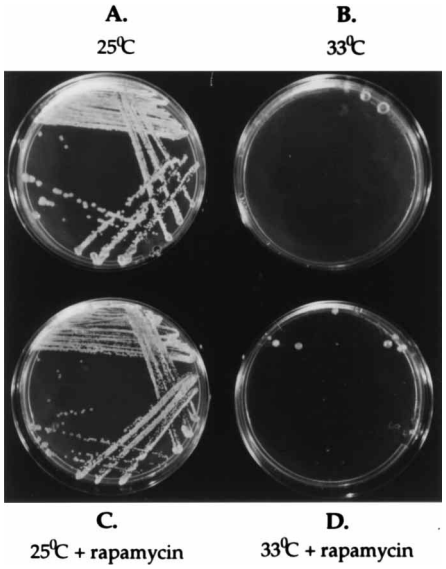

FIG. 9. Rapamycin does not rescue the *pat1-114* growth defect. *pat1-114 h*$^+$ cells were streaked on EMM in the presence of the drug vehicle (A and B) or rapamycin (C and D). Incubation was either at 25 or 33°C. Rapamycin did not suppress the lethal phenotype of *pat1-114* cells at the restrictive temperature.

DISCUSSION

Rapamycin specifically inhibits sexual development in response to starvation. We report here, for the first time, the effect of rapamycin on fission yeast. We found that in contrast to its effect on *S. cerevisiae* and other fungi, rapamycin does not exhibit any apparent effect on the vegetative growth of *S. pombe*. Instead, the effect of the drug becomes apparent only upon starvation. Under conditions of nutrient deprivation wild-type homothallic cells can either enter the sexual development pathway or the stationary phase. Rapamycin specifically inhibits the sexual development pathway before the stage of agglutination and does not affect entry into the stationary phase. Our conclusion that rapamycin inhibits sexual development at a very early step is supported by several observations. First, cellular agglutination, one of the first discernible stages in the conjugation process, is inhibited by rapamycin. Second, zygote formation is inhibited by rapamycin, as observed in standard mating assays. And third, rapamycin has only slight effect, or no effect at all, on the sporulation of diploid cells, indicating that later stages of sexual development, that is, entrance into meiosis and spore formation, are far less affected by rapamycin.

The lack of effect of rapamycin during vegetative growth does not reflect an inability of the drug to penetrate vegetatively growing cells. Our findings that cells are rendered sterile if they are grown in the presence of rapamycin (and then starved in the absence of the drug) indicate that the drug enters the growing cells, but its effect becomes apparent only upon starvation. It is possible that the target of rapamycin exists in vegetatively growing cells but that it is activated or becomes effective only upon starvation. It is noteworthy that the most pronounced effect, more than a 500-fold reduction in mating efficiency, is observed when rapamycin is present during both growth and starvation.

The inhibition of rapamycin on sexual development is not mating type specific. When only one mating type was grown with the drug and was then mixed with an untreated opposite mating type, the resulting mating efficiency was independent of the mating type which had been grown with the drug. Interestingly, the resulting mating efficiency of such crosses was intermediate; that is, it was lower (by 2.5-fold) than the mating efficiency of untreated cells but higher (by 4-fold) than the mating efficiency of crosses in which both mating types were grown in the presence of rapamycin. Thus, it appears that the untreated cells can partially suppress the effect of rapamycin.

The effect of rapamycin on sexual development mutants. We have initiated our investigations on the mode of action of rapamycin by examining the effect of rapamycin on sexual development mutants. Our findings are all in agreement with the hypothesis that rapamycin acts at a very early stage in the sexual development pathway. However, the specific pathway(s) or protein(s) affected by rapamycin remains unknown. A central element in the sexual differentiation pathway is the *ras1*$^+$ gene. The *ras1*$^+$ gene product is essential for both mating and meiosis but is dispensable for vegetative growth. Like its mammalian counterpart, the *ras1*$^+$ gene product participates in the regulation of a protein kinase cascade that ultimately regulates a member of the mitogen-activated protein kinase family (29, 57, 75). Our studies show that the sterility associated with rapamycin is not suppressed by overexpression of the wild-type *ras1*$^+$ gene. However, expression of the *ras1*$^+$ activated allele, *ras1*^{Val17}, restores the ability of the rapamycin-treated cells to agglutinate and respond to mating conditions, as demonstrated by the development of conjugation tubes upon starvation, independent of the presence of the drug. Therefore, it is clear that rapamycin does not exert its effect downstream of the *ras1*$^+$ gene product. The simplest explanation for our data is that the target of rapamycin acts upstream of the *ras1*$^+$ gene product, either directly or indirectly. An alternative explanation is that rapamycin acts on a pathway which is parallel to the *ras1* pathway and which shares with the *ras1* pathway some of its functions. For example, mating signals are also mediated by the G protein α subunit, gpa1. The gpa1-mediated signals are transmitted parallel to those in the *ras1* pathway and converge on byr2 (78).

In view of the findings in other model systems, a direct effect of rapamycin upstream of the *ras1*$^+$ gene product is less likely. In mammalian cells, neither Ras nor Raf appears to be part of a pathway which is affected by rapamycin (14, 20, 53). The RAS proteins of *S. cerevisiae* are also unlikely to mediate the rapamycin effect. This suggestion is based on the findings that constitutively activated mutants of the RAS/cAMP pathway in *S. cerevisiae*, including those resulting from the expression of the dominant activated *RAS2* allele, *RAS2*^{Val19}, do not suppress the growth arrest induced by rapamycin (3). The genetic relationships between the pathway which mediates the rapamycin effect and the *ras1*$^+$ pathway remain to be determined.

cAMP plays an important role in the regulation of sexual differentiation in *S. pombe*. The addition of cAMP to the medium or gene mutations which lead to an increase in cAMP level or pka1 activity inhibit sexual differentiation. Our findings do not support the hypothesis that rapamycin inhibits sexual differentiation by stimulation of the cAMP/pka1 pathway. First, rapamycin does not suppress the unscheduled meiosis of *pat1-114* mutants. In contrast, this lethal phenotype is suppressed by processes which raise the cAMP intracellular level, such as the introduction of cAMP into the growth medium and the overexpression of *pka1*$^+$ (49). In addition, elevated cAMP levels can cause an impairment in the normal control of cell growth. For example, loss of function of the regulatory subunit of the cAMP-dependent protein kinase (cgs1) inhibits mating and meiosis, but also brings about an increase in cell length at division (18). Rapamycin, on the other hand, does not exert any detectable effects on cell growth or cell size. Furthermore, rapamycin does not exert its effect downstream of the cAMP-dependent protein kinase. This was shown by the inability of rapamycin to inhibit the derepression of sexual development of mutants carrying defective adenylate cyclase or protein kinase A catalytic subunit genes.

Possible mechanisms for the inhibitory effect of rapamycin on sexual development. Rapamycin strongly inhibits sexual conjugation of haploid cells, a process which requires conditions of starvation. In contrast, two other processes which are affected by starvation, that is, entrance into meiosis and entrance into stationary phase, are only slightly affected or not affected at all by rapamycin. Thus, it appears that cells are able to sense nutrient depletion, but the switch from mitotic cycles to sexual development in haploid cells is specifically inhibited by rapamycin. One possible explanation for the sterility effect is that rapamycin inhibits the expression of specific genes which are required early in the sexual differentiation process, such as the genes required for pheromone production or response. However, the slight effect of rapamycin on the sporulation of diploids seems to argue against a direct effect of rapamycin on pheromone production, because the process of meiosis requires an intact pheromone signalling pathway (reviewed in reference 79). Nevertheless, conjugation is more sensitive to pheromone activities than meiosis (46). Hence, it is possible that rapamycin causes a reduction in the mating pheromone signals but that this reduction leads to a far more dramatic effect on mating than on meiosis.

The effect of rapamycin on *S. pombe* cells appears at first sight to be substantially different than its effects on other cell systems, as it does not affect cell cycle progression. However, a close look at the regulation of the cell cycle in *S. pombe* may indeed suggest that the effect of rapamycin is caused by inhibition of a function in early G_1, before Start. This inhibition may become apparent only upon starvation. In *S. pombe*, the G_1 phase is particularly short in rapidly growing cells. Under rapid-growth conditions the G_1-S size control is cryptic, as wild-type cells at the end of mitosis are already beyond the minimum size for entry into S phase (reviewed in reference 44). Therefore, inhibition of one or more functions required for control in G_1, specifically, functions which delay cell cycle progression during this phase, may not cause an effect during rapid growth. In contrast, the G_1 control becomes limiting under conditions in which mitosis is initiated at a reduced size, such as during nitrogen starvation. A delay in G_1 is necessary under starvation conditions to allow not only the accumulation of enough mass but also the initiation of processes required for mating. Thus, the sterility of rapamycin-treated cells may indeed be the result of inhibition of a function(s) which normally enables the cells to linger in G_1. In this regard the phenotype of *rum1* mutants in *S. pombe* may be relevant. The *rum1*$^+$ gene has a central role in the regulation of G_1 progression. It encodes a stoichiometric inhibitor of cdc2, the central CDK which controls both G_1-S and G_2-M transitions. Cells with *rum1* deleted exhibit no defect during vegetative growth but are profoundly sterile (50). Rapamycin may cause an effect similar to that of *rum1* mutants. This hypothesis is currently being examined in our laboratory.

ACKNOWLEDGMENTS

We thank P. A. Fantes and S. A. MacNeill (University of Edinburgh, Edinburgh, United Kingdom) for *S. pombe* strains, A. Cohen (Hebrew University, Jerusalem, Israel) for the SP837 strain, D. Hughes (Chester Beatty Laboratories, London, United Kingdom) for *ras1* and *ste6* plasmids, and C. S. Hoffman (Boston College, Chestnut Hill, Mass.) for *git2* and *git6* mutants. We are grateful to M. Mevarech (Tel Aviv University, Tel Aviv, Israel) for encouraging us to begin this work and for helpful discussions and to P. A. Fantes (University of Edinburgh) for useful comments during the course of this work.

This work was supported partly by the Israeli Ministry of Health.

REFERENCES

1. **Abraham, R. T., and G. J. Wiederrecht.** 1996. Immunopharmacology of rapamycin. Annu. Rev. Immunol. **14:**483–510.
2. **Albers, M. W., R. T. Williams, E. J. Brown, A. Tanaka, F. L. Hall, and S. L. Schreiber.** 1993. FKBP-rapamycin inhibits a cyclin-dependent kinase activity and a cyclin D1-Cdk association in early G1 of an osteosarcoma cell line. J. Biol. Chem. **268:**22825–22829.
3. **Barbet, N. C., U. Shneider, S. B. Helliwell, I. Stansfield, M. F. Tuite, and M. N. Hall.** 1996. TOR controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell **7:**25–42.
4. **Berreta, L., A.-C. Gingras, Y. Svitkin, M. N. Hall, and N. Sonenberg.** 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J. **15:**658–664.
5. **Bierer, B. E., P. S. Mattila, R. F. Standaert, L. A. Herzenberg, S. J. Burakoff, G. Crabtree, and S. L. Schreiber.** 1990. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proc. Natl. Acad. Sci. USA **87:**9231–9235.
6. **Brazelton, T. R., and R. E. Morris.** 1996. Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr. Immunol. **8:** 710–720.
7. **Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber.** 1994. A mammalian protein targeted by G1 arresting rapamycin-receptor complex. Nature **369:**756–758.
8. **Brown, E. J., P. A. Beal, C. T. Keith, J. Chen, T. B. Shin, and S. L. Schreiber.** 1995. Control of p70 S6 kinase by kinase activity of FRAP in vivo. Nature **377:**441–446.
9. **Brunn, G. J., J. Williams, C. Sabers, G. Wiederrecht, J. C. Lawrence, Jr., and R. Abraham.** 1996. Direct inhibition of the signalling function of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J. **15:**5256–5267.
10. **Cafferkey, R., P. R. Young, M. M. McLaughlin, D. J. Bergsma, Y. Koltin, G. M. Sathe, L. Faucette, W.-K. Eng, R. K. Johnson, and G. P. Livi.** 1993. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol. Cell. Biol. **13:**6012–6023.
11. **Calvo, V., C. M. Crews, T. A. Vik, and B. E. Bierer.** 1992. Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppressant rapamycin. Proc. Natl. Acad. Sci. USA **89:**7571–7575.
12. **Cardenas, M. E., R. S. Muir, T. Breuder, and J. Heitman.** 1995. Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. EMBO J. **14:**2772–2783.
13. **Chiu, M. I., H. Katz, and V. Berlin.** 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proc. Natl. Acad. Sci. USA **91:**12574–12578.
14. **Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis.** 1992. Rapamycin-FKBP specifically blocks growth dependent activation of the signaling by the 70 kd S6 protein kinases. Cell **69:**1227–1236.
15. **Clipstone, N. A., and G. R. Crabtree.** 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature **357:**695–697.
16. **Costello, G., L. Rodgers, and D. Beach.** 1986. Fission yeast enters the stationary phase G_0 state from either mitotic G1 or G2. Curr. Genet. **11:**119–125.
17. **de Groot, R. P., L. M. Ballou, and P. Sassone-Corsi.** 1994. Positive regulation of the cAMP-responsive activator CREM by p70 S6 kinase: an alternative route to mitogen induced gene expression. Cell **79:**81–91.
18. **DeVoti, J., G. Seydoux, D. Beach, and M. McLeod.** 1991. Interactions between *ran1*$^+$ protein kinase and cAMP dependent protein kinase as negative regulators of fission yeast meiosis. EMBO J. **10:**3759–3768.
19. **Di Como, C. J., and K. T. Arndt.** 1996. Nutrients, via the Tor proteins, stimulate the association of TAP42 with type 2A phosphatases. Genes Dev. **10:**1904–1916.
20. **Downward, J.** 1994. Regulating S6 kinase. Nature **371:**378–379.
21. **Dumont, F. J., M. J. Staruch, S. J. Koprak, M. R. Melino, and N. H. Sigal.** 1990. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK506 and rapamycin. J. Immunol. **144:**251–258.
22. **Egel, R.** 1989. Mating type genes, meiosis and sporulation, p. 31–68. *In* A. Nasim, P. Young, and B. F. Johnston (ed.), Molecular biology of the fission yeast. Academic Press, New York, N.Y.
23. **Ferrari, S., R. B. Pearson, M. Siegmann, S. C. Kozma, and G. Thomas.** 1993. The immunosuppressant rapamycin induces inactivation of $p70^{S6k}$ through dephosphorylation of a novel set of sites. J. Biol. Chem. **268:**16091–16094.
24. **Feuerstein, N., D. Huang, and M. B. Prystowsky.** 1995. Rapamycin selectively blocks interleukin-2-induced proliferating cell nuclear antigen gene expression in T lymphocytes. Evidence for inhibition of CREB/ATF binding activities. J. Biol. Chem. **270:**9454–9458.
25. **Foor, F., S. A. Parent, N. Morin, A. M. Dahl, N. Ramadan, G. Chrebet, K. A. Bostian, and J. B. Nielsen.** 1992. Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α-factor arrest in yeast. Nature **360:**682–684.
26. **Fukui, Y., Y. Kaziro, and M. Yamamoto.** 1986. Mating pheromone-like diffusible factor released by *Schizosaccharomyces pombe*. EMBO J. **5:**1991–1993.
27. **Fukui, Y., T. Kozasa, Y. Kaziro, T. Takeda, and M. Yamamoto.** 1986. Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. Cell **44:** 329–336.
28. **Galat, A.** 1993. Peptidyl-prolyl *cis trans* isomerases: immunophilins. Eur. J. Biochem. **216:**689–707.
29. **Gotoh, Y., E. Nishida, M. Shimanuki, T. Toda, Y. Imai, and M. Yamamoto.** 1993. *Schizosaccharomyces pombe* Spk1 is a tyrosine-phosphorylated protein functionally related to *Xenopus* mitogen-activated protein kinase. Mol. Cell. Biol. **13:**6427–6434.
30. **Gutz, H., H. Heslot, U. Leupold, and N. Loperino.** 1974. *Schizosaccharomyces pombe*, p. 395–446. *In* R. D. King (ed.), Handbook of genetics, vol. 1. Plenum Publishing Corporation, New York, N.Y.
31. **Heitman, J., N. R. Movva, and M. N. Hall.** 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science **253:**905–909.
32. **Helliwell, S., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M. Hall.** 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol. Biol. Cell **5:**105–118.
33. **Hughes, D. A., and M. Yamamoto.** 1993. Prokaryotic and simple eukaryotic systems, p. 123–146. *In* J. Kurjan and B. L. Taylor (ed.), Signal transduction. Academic Press, New York, N.Y.
34. **Hughes, D. A., Y. Fukui, and M. Yamamoto.** 1990. Homologous activators of ras in fission and budding yeast. Nature **344:**355–357.
35. **Iino, Y., and M. Yamamoto.** 1985. Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **82:**2447–2451.
36. **Jefferies, H. B. J., C. Reinhard, S. C. Kozma, and G. Thomas.** 1994. Rapa-

mycin selectively represses translation of the 'polypyrimidine tract' mRNA family. Proc. Natl. Acad. Sci. USA **91:**4441–4445.
37. **Kitamura, K., T. Nakagawa, and C. Shimoda.** 1990. Novel sterile mutants of fission yeast *Schizosaccharomyces pombe* which are defective in their response to starvation. Curr. Genet. **18:**315–321.
38. **Kohli, J., H. Hottinger, P. Munz, A. Strauss, and P. Thuriaux.** 1977. Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidisation. Genetics **87:**471–489.
39. **Koltin, Y., L. Faucette, D. J. Bergsma, M. A. Levy, R. Cafferkey, P. L. Koser, R. K. Johnson, and G. P. Livi.** 1991. Rapamaycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl *cis-trans* isomerase related to human FK506-binding protein. Mol. Cell. Biol. **11:**1718–1723.
40. **Koser, P. L., D. J. Bergsma, R. Cafferkey, W.-K. Eng, N. M. McLaughlin, A. Ferrara, C. Silverman, K. Kasyan, M. J. Bossard, R. K. Johnson, T. G. Porter, M. A. Levy, and G. P. Livi.** 1991. The *CYP2* gene of *Saccharomyces cerevisiae* encodes a cyclosporin A peptidyl prolyl *cis trans* isomerase with N-terminal signal sequence. Gene **108:**74–80.
41. **Kunz, J., and M. N. Hall.** 1993. Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. Trends Biochem. **18:**334–338.
42. **Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall.** 1993. Cell **73:**585–596.
43. **Kuo, C. J., J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree.** 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature **358:**70–73.
44. **Labib, K., and S. Moreno.** 1996. rum1: a CDK inhibitor regulating G1 progression in fission yeast. Trends Cell Biol. **6:**62–65.
45. **Leupold, U.** 1955. Metodisches zur genetik von *Schizosaccharomyces pombe*. Schweiz. Z. Allg. Pathol. Bakteriol. **18:**1141–1146.
46. **Leupold, U., M. Sipiczki, and R. Egel.** 1991. Pheromone production and response in sterile mutants of fission yeast. Curr. Genet. **20:**79–85.
47. **Lin, T.-A., X. Kong, A. R. Saltiel, P. J. Blackshear, and J. C. Lawrence, Jr.** 1995. Control of PHAS-I by insulin in 3T3-L1 adipocytes. J. Biol. Chem. **270:**18531–18538.
48. **MacNeill, S. A., E. Warbrick, and P. A. Fantes.** 1991. Controlling cell cycle progress in the fission yeast *Schizosaccharomyces prombe*. Curr. Opin. Genet. Dev. **1:**307–312.
49. **Maeda, T., Y. Watanabe, H. Kunitomo, and M. Yamamoto.** 1994. Cloning of the *pka1* gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. J. Biol. Chem. **269:**9632–9637.
50. **Martin-Castellanos, C., K. Labib, and S. Moreno.** 1996. B-type cyclins regulate G1 progression in fission yeast in opposition to the $p25^{rum1}$ cdk inhibitor. EMBO J. **15:**839–849.
51. **McCaffrey, P. G., B. A. Perrino, T. R. Soderling, and A. Rao.** 1993. NF-ATp, a T-lymphocyte DNA binding protein that is a target for calcineurin and immunosuppressive drugs. J. Biol. Chem. **268:**3747–3752.
52. **McLeod, M., and D. Beach.** 1988. A specific inhibitor of the $ran1^{+}$ protein kinase regulates entry into meiosis in *Schizosaccharomyces pombe*. Nature **332:**509–514.
53. **Ming, X.-F., B. M. T. Burgering, S. Wenström, L. Claesson-Welsh, C.-H. Heldin, J. L. Bos, and G. Thomas.** 1994. Activation of p70/p85 S6 kinase by a pathway independent of $p21^{ras}$. Nature **371:**426–429.
54. **Molnar, M., and M. Sipiczki.** 1995. Two novel genes involved in the sexual development of *Schizosaccharomyces pombe*. Curr. Genet. **28:**447–453.
55. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:**795–823.
56. **Morice, W. G., G. Weiderrecht, G. J. Brunn, J. J. Siekierka, and R. T. Abraham.** 1993. Rapamycin inhibition of interleukin-2-dependent $p33^{cdk2}$ and $p34^{cdc2}$ kinase activation in T lymphocytes. J. Biol. Chem. **268:**22737–22745.
57. **Nadin-Davis, S. A., and A. Nasim.** 1990. *Schizosaccharomyces pombe ras1* and *byr1* are functionally related genes of the *ste* family that affect starvation-induced transcription of mating-type genes. Mol. Cell. Biol. **10:**549–560.
58. **Nadin-Davis, S. A., A. Nasim, and D. Beach.** 1986. Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. EMBO J. **5:**2963–2971.
59. **Nielsen, O., J. Davey, and R. Egel.** 1992. The *ras1* function of *Schizosaccharomyces pombe* mediates pheromone induced transcription. EMBO J. **11:**1391–1395.
60. **Nourse, J., E. Firpo, W. M. Flanagan, S. Coats, K. Polyak, M.-H. Lee, J. Massague, G. R. Crabtree, and J. M. Roberts.** 1994. Interleukin-2-mediated elimination of the $p27^{kip1}$ cyclin dependent kinase inhibitor prevented by rapamycin. Nature **372:**570–573.
61. **Nurse, P.** 1985. Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. Mol. Gen. Genet. **198:**497–502.
62. **Nurse, P.** 1990. Universal control mechanism regulating onset of M-phase. Nature **344:**503–508.
63. **Prentice, H.** 1992. High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. **20:**621.
64. **Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer.** 1992. Rapamycin induced inhibition of the 70-kilodalton S6 protein kinase. Science **257:**973–977.
65. **Russell, P., and P. Nurse.** 1986. *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*: a look at yeasts divided. Cell **45:**781–782.
66. **Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder.** 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin dependent fashion and is homologous to yeast TORs. Cell **78:**35–43.
67. **Sabers, C. J., G. Wiederrecht, J. M. Williams, M. M. Martin, F. J. Dumont, and R. T. Abraham.** 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. **270:**815–822.
68. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
69. **Schreiber, S. L., and G. R. Crabtree.** 1992. The mechanism of action of cyclosporin A and FK506. Immunol. Today **13:**136–142.
70. **Sigal, N. H., and F. J. Dumont.** 1992. Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocytes and signal transduction. Annu. Rev. Immunol. **10:**519–560.
71. **Sipiczki, M.** 1989. Taxonomy and phylogenesis, p. 431–448. *In* A. Nasim, P. Young, and B. F. Johnston (ed.), Molecular biology of the fission yeast. Academic Press, New York, N.Y.
72. **Stettler, S., E. Warbrick, S. Prochnik, S. Mackie, and P. Fantes.** 1996. The wis1 signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast. J. Cell Sci. **109:**1927–1935.
73. **Terada, N., H. R. Patel, K. Takase, K. Kohno, A. C. Nairn, and E. W. Gelfand.** 1994. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc. Natl. Acad. Sci. USA **91:**11477–11481.
74. **Tropschug, M., I. B. Barthelmess, and W. Newpert.** 1989. Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. Nature **342:**953–955.
75. **Wang, Y., H.-P. Xu, M. Riggs, L. Rodgers, and M. Wigler.** 1991. *byr2*, a *Schizosaccharomyces pombe* gene encoding a protein kinase capable of partial suppression of the *ras1* mutant phenotype. Mol. Cell. Biol. **11:**3554–3563.
76. **Weisman, R., J. Creanor, and P. Fantes.** 1995. A multicopy suppressor of a cell cycle defect in *S. pombe* encodes a heat shock inducible 40 kDa cyclophilin-like protein. EMBO J. **15:**447–456.
77. **Wu, L., and P. Russell.** 1997. Roles of Wee1 and Nim1 protein kinases in regulating the switch from mitotic division to sexual development in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **17:**10–17.
78. **Xu, H.-P., M. White, S. Marcus, and M. Wigler.** 1994. Concerted action of RAS and G proteins in the sexual response pathways of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **14:**50–58.
79. **Yamamoto, M.** 1996. The molecular mechanisms of meiosis in fission yeast. Trends Biochem. Sci. **21:**18–22.
80. **Yoshida, T., T. Toda, and M. Yanagida.** 1994. Characterization of calcineurin-like phosphatase gene $ppb1^{+}$ in fission yeast: null mutation and cyclosporin A cause similar cell cycle inhibition. J. Cell Sci. **107:**1725–1735.