Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast

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Simultaneous inactivation of ppy1 and ppy2 PTPases in fission yeast leads to aberrant cell morphology and growth arrest. Spontaneous recessive mutations that bypass the requirement for ppy1 and ppy2 and reside in two complementation groups were isolated, sty1 and sty2. sty1⁻ and sty2⁻ mutant cells are substantially delayed in the timing of mitotic initiation. We have isolated the sty1 gene, which encodes a MAP kinase that is closely related to a subfamily of MAP kinases regulated by osmotic stress including Saccharomyces cerevisiae HOG1 and human CSBP1. We find that sty2 is allelic to the wis1 MAP kinase kinase and that Δsty1 and Δwis1 cells are unable to grow in high osmolarity medium. Osmotic stress induces both tyrosine phosphorylation of Sty1 and a reduction in cell size at division. Pyp2 associates with and tyrosine dephosphorylates Sty1 in vitro. We find that wis1-dependent induction of pyp2 mRNA is responsible for tyrosine dephosphorylation of Sty1 in vivo on prolonged exposure to osmotic stress. We conclude that Pyp1 and Pyp2 are tyrosine-specific MAP kinase phosphatases that inactivate an osmoregulated MAP kinase, Sty1, which acts downstream of the Wis1 MAP kinase kinase to control cell size at division in fission yeast.

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Growing fission yeast cells tend to divide at a constant size at division. The fission yeast cell cycle is controlled at two major points, in G₁ at entry into S phase (DNA replication) and at the initiation of mitosis. Genetic and physiological studies have revealed that the timing of both transitions requires attainment of a critical cell size and has lead to the proposal that mass sensors govern these cell cycle transitions (Nurse 1975; Fantes and Nurse 1977). Under conditions of normal growth the mass sensor governing the initiation of DNA synthesis is cryptic, such that cell size at division is rate-limiting for cell cycle progression (Fantes and Nurse 1977). Because Schizosaccharomyces pombe cells grow by length extension, this is expressed as the attainment of a critical cell length. Cell size at division can be influenced either by the genotype of the cell or by the prevailing nutritional conditions. The molecular nature of the mass sensors governing these transitions is not known.

A considerable advance in our understanding of the molecular basis of mitotic initiation, nevertheless, has come from the analysis of mutants in fission yeast that uncouple size control from cell cycle progression. In particular the cell division cycle (cdc) mutants have been particularly valuable in identifying the central components of this control (Nurse et al. 1976; Thuriaux et al. 1978; Nasmyth and Nurse 1981). It is now recognized that the timing of mitotic initiation is controlled by rapid tyrosine dephosphorylation and activation of the catalytic kinase subunit of the Cdc13 (cyclin B)/Cdc2 kinase complex by the Cdc25 phosphatase (for review, see Millar and Russell 1992). This complex is maintained in an inactivated state during interphase by the action of the wee1 and mik1 tyrosine kinases (Russell and Nurse 1987a; Lundgren et al. 1991). Structural and functional homologs of the cdc2, cdc13, cdc25, and wee1 genes have been shown to exist in metazoa supporting the view that entry into mitosis is controlled by a universally conserved mechanism (Nurse 1990).

Extensive genetic analysis in S. pombe has revealed several other sets of genes that show genetic interaction with the core elements described above and, by inference, also encode gene products that control the timing of mitosis. These include win1 and five multicopy suppressors of win1, wis1–wis5 (Ogden and Fantes 1986; Warbrick and Fantes 1991, 1992), the nim1/cdr1 and cdr2 genes (Russell and Nurse 1987b; Young and Fantes 1987), and six mitotic catastrophe suppressors mcs1–mcs6 (Molz et al. 1989). The Nim1 kinase acts to inhibit the Wee1 tyrosine kinase by direct phosphorylation (Coleman et al. 1993; Parker et al. 1993; Wu and Russell 1993). The wis1 gene, which acts as a dose-dependent initiator of mitosis, encodes a protein that is homologous to a family of MAP kinase kinases (Ogden and Fantes 1986; Warbrick and Fantes 1991).
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This latter observation is particularly intriguing because MAP kinase cascades exist in all eucaryotic cells and have been implicated in a wide range of cellular processes including mitogenic signal transduction, cellular differentiation, and response to environmental stress (for review, see Ammerer 1994; Davis 1994; Marshall 1994; Herskowitz 1995). At least four distinct MAP kinase pathways have been identified in the unicellular budding yeast Saccharomyces cervisiae, which are required for mating, sporulation, control of cell polarity, and the cellular response to osmotic stress. Signal transduction through MAP kinase cascades involves sequential phosphorylation and activation of three distinct kinases: the MAP kinase kinase kinase (or MEKK), the MAP kinase kinase (or MEK), and the MAP kinase itself. Although there appear to be multiple mechanisms by which plasma membrane-associated receptors can induce activation of MEKK, it has now been demonstrated clearly that MEKK activation leads to MAP kinase kinase activation by direct phosphorylation. The MAP kinase kinase in turn activates the MAP kinase by dual phosphorylation on two closely spaced residues, a threonine and a tyrosine. Less is understood of the mechanisms that terminate signal transduction through MAP kinase cascades, although inactivation of MAP kinases has been ascribed to the action of dual specificity phosphatases that remove both of the phosphates from these residues (Alessi et al. 1993; Sun et al. 1993; Doi et al. 1994; Ward et al. 1994).

We and others recently have identified two additional components of fission yeast mitotic control, pyp1 and pyp2, which encode tyrosine-specific phosphatases. Overexpression of either pyp1 or pyp2 causes a delay in mitotic initiation. Conversely, disruption of pyp1 and, to a lesser extent, pyp2 causes cells to divide at a reduced cell size. Simultaneous disruption of these genes is lethal, demonstrating that pyp1 and pyp2 share an essential overlapping function (Millar et al. 1992b; Ottilie et al. 1991, 1992). We have constructed a conditional double mutant and undertaken a suppression analysis to investigate the role of pyp1 and pyp2 in the fission yeast cell cycle. We demonstrate that the pyp1 and pyp2 PTPases regulate a MAP kinase cascade that controls the timing of mitotic initiation in fission yeast. We have identified the central components of this pathway as the the wts1 MAP kinase kinase and a novel MAP kinase, encoded by the sty1 gene. We show that the sty1 MAP kinase pathway is regulated by changes in external osmolarity. These studies provide an important link between the cellular response to environmental stress and control of the cell cycle.

Results

Inactivation of Pyp1 and Pyp2 causes aberrant cell morphology and growth arrest

Inactivation of either the pyp1 or pyp2 tyrosine-specific phosphatases leads to a reduction in cell size at division in fission yeast. Simultaneous disruption of pyp1 and pyp2 gives rise to inviable spores that do not germinate (Millar et al. 1992b). To examine the role of pyp1 and pyp2 in the mitotic cell cycle in fission yeast, a conditional allele of pyp1 under the control of the thiamine-repressible nmt1 promoter was constructed (see Materials and methods). In the absence of thiamine NMT–pyp1 cells divided at 14.3±0.4 μm, a size similar to wild-type cells, whereas in the presence of thiamine, cells divided at 11.1±0.3 μm, similar to Δpyp1 cells (Millar et al. 1992b). To examine the effects of a loss of pyp1 expression in a Δpyp2 strain, NMT–pyp1 Δpyp2 cells were grown in the absence of thiamine and then streaked onto minimal medium in the presence of 10 μM thiamine. In contrast to wild-type, NMT–pyp1 or Δpyp2 cells, NMT–pyp1 Δpyp2 cells were greatly retarded for growth on medium containing thiamine (Fig. 1A). Microscopic ex-

![Figure 1](https://example.com/image1.png)

**Figure 1.** Suppression of pyp1 and pyp2 expression leads to abnormal cell morphology. (A) Cells were grown on minimal medium lacking thiamine and then streaked onto the same medium in the absence (left plate) or presence of 10 μM of thiamine (right plate) and allowed to grow for 3 days at 25°C. The genotypes of the cells were wild-type strain 972 (top left-hand segment), pyp2::LEU2 (top right-hand segment), NMT–pyp1(ura4) [bottom left-hand segment], and NMT–pyp1(ura4) pyp2::LEU2 (bottom right-hand segment). On prolonged incubation NMT–pyp1 Δpyp2 cells eventually recovered, presumably because of metabolic depletion of thiamine in the medium. (B) The phenotype of NMT–pyp1(ura4) pyp2::LEU2 cells was examined by phase-contrast microscopy after 3 days growth on minimal medium either in the absence (left) or presence of 10 μM of thiamine (right).
amination revealed that these cells arrested after three or four divisions as large, spherical, swollen cells, a proportion of which lysed [Fig. 1B]. In the absence of thiamine, cells were indistinguishable from wild type [Fig. 1B]. Growth arrest in the presence of thiamine was not suppressed by the presence of 1 M sorbitol, an osmotic stabilizing agent [data not shown]. We conclude that the pypl and pypp2 genes, in addition to control of mitotic initiation, are required for cell shape control in fission yeast.

**syl1 and syl2 mutants suppress a pypl−pypp2− growth defect**

We utilized the conditional arrest of NMT−pypl Δpypp2 cells to isolate mutations that bypass the requirement for the pypl and pypp2 PTPases by pseudoreversion analysis. From a total of 107 cells plated, 56 fast-growing colonies were identified, of which 17 grew in the absence of thiamine. Cells from all 17 mutant strains were highly elongated relative to wild-type [see below]. When out-crossed to a wild type strain the elongated phenotype was found to be unlinked to either the NMT−pypl or Δpypp2 alleles. Additionally, cross-mating of these strains revealed that these 17 mutants fell into two complementation groups, syl1 [11 alleles] and syl2 [6 alleles] [for suppressor of tyrosine phosphatase]. Because all mutants within each complementation group appeared to be elongated to the same extent, a single mutant from each complementation group, syl1-1 and syl2-1, was chosen for further study. Further crosses revealed that both the syl1-1 and syl2-1 mutations segregated 2:2 with respect to the elongated phenotype, indicating that both were the consequence of a single nuclear lesion. To determine whether the syl1-1 and syl2-1 mutations could completely bypass the requirement for pypl and pypp2, syl1-1 pypp2::LEU2 or syl2-1 pypp2::LEU2 cells were crossed to pypl1::ura4 cells. Spores from both crosses gave rise to colonies of elongated cells that were able to grow in the absence of both uracil and leucine indicating that they had inherited both Δpypl and Δpypp2 alleles. Southern blot analysis was used to confirm the genotypes of the syl1-1 Δpypl Δpypp2 and syl2-1 Δpypl Δpypp2 triple mutants [data not shown]. In a control cross, pypp2::LEU2 was mated to pypl1::ura4. From >3000 spores analyzed, no colonies grew in the absence of both uracil and leucine, confirming previous observations that simultaneous loss of pypl and pypp2 function is lethal [Millar et al. 1992b; Ottilie et al. 1992]. These results suggest that both the syl1-1 and syl2-1 mutations were able to bypass the synthetic lethal combination of a Δpypl Δpypp2 double mutant.

**syl1 and syl2 mutants are delayed in mitotic initiation**

The role of syl1 and syl2 in the mitotic cell cycle was examined. Growth rates of syl1-1 or syl2-1 cells were found to be identical to wild type with a doubling time of 2.5 hr in rich medium, or 3.5 hr in synthetic minimal medium at 30°C, indicating that neither mutant was defective in cellular growth rate or division. However, when grown to mid-log phase, syl1-1 and syl2-1 mutant cells were found to be substantially longer at division than the wild-type control [Table 1], suggesting that they are delayed in the timing of mitotic initiation. These cells initiate mitosis at a size similar to cdc25-22 cells, which express a partially defective cdc25 phosphatase [Table 1]. To investigate further the role of syl1 and syl2 in mitotic control, syl1-1 cells were crossed to cdc25-22 cells and random spore analysis performed. Approximately 25% of the spores from this cross germinated on YEPD plates at 26°C but arrested after one or two divisions as highly elongated cells, suggesting that the syl1-1 cdc25-22 double mutants are synthetically lethal. To confirm this, ascii from the same cross were subjected to tetrad dissection. The expected number of PD, TT, and NPD were observed, indicating that cdc25 and syl1 are unlinked genes. At least five NPD tetrads from the same cross had two spores that gave rise to wild-type colonies and two that underwent cell cycle arrest at 26°C [Table 1]. The inviable cells from these NPDs were classified with the genotype syl1-1 cdc25-22. Idential results were found when crossing syl2-1 to cdc25-22 [Table 1]. These data indicate that the products of the syl1 and syl2 genes are required for the control of cell size at division in fission yeast.

**syl1 and syl2 mediate mitotic control by the pypl and pypp2 PTPases**

We have shown previously that overexpression of pypl or pypp2 causes a delay in the timing of mitosis, whereas disruption of either gene has the opposite effect [Ottilie et al. 1991, 1992; Millar et al. 1992b]. Two lines of evidence suggest that syl1 and syl2 mediate control of cell size at division by the pypl and pypp2 PTPases. First, syl1-1 Δpypl Δpypp2 and syl2-1 Δpypl Δpypp2 cells initiate cell division at the same size as syl1-1 and syl2-1 single mutants alone [Table 1]. Second, no additional delay in mitotic initiation was observed when syl1-1 and

| Temperature [°C] | Cell size at division [μm] |
|-----------------|---------------------------|
| Wild type       | 30                        | 14.2 ± 0.3               |
| cdc25-22        | 26                        | 21.9 ± 0.8               |
| syl1-1          | 30                        | 23.2 ± 1.6               |
| syl1-1 pypl1::ura4 pypp2::LEU2 | 30                     | 22.9 ± 2.1              |
| syl1-1 cdc25-22 | 26                        | cdc                      |
| syl2-1          | 30                        | 23.4 ± 1.3               |
| syl2-1 pypl1::ura4 pypp2::LEU2 | 30                     | 24.2 ± 1.3              |
| syl2-1 cdc25-22 | 26                        | cdc                      |

Cell size measurements of septated cells grown in either liquid synthetic minimal medium, [EMM]. [cdc] Cell division cycle phenotype. The temperature at which the measurements were made is indicated. Measurements were the mean of 30 individual determinations (±S.D.).

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sty2-1 cells were transformed with a plasmid constitutively expressing either pyp1 or pyp2 from the strong adh promoter as compared with empty vector alone (data not shown). From this we conclude that the sty1-1 and sty2-1 cells are completely epistatic to control of mitotic initiation by the pyp1 and pyp2 PTPases.

sty2 is allelic to the wis1 MAP kinase kinase

We noted that mutants in the wis1 gene, which encodes a putative MAP kinase kinase homolog, are delayed in mitotic initiation to a similar extent to the sty1-1 and sty2-1 mutants [Warbrick and Fantes 1991]. The relationship of wis1 to sty1 and sty2 was examined. During initial characterization we observed that the growth of single sty1-1 and sty2-1 mutant cells was defective at high temperatures. Neither sty1-1 nor sty2-1 mutant cells were able to grow at 36.5°C, whereas wild-type cells are unaffected [Fig. 2A]. Importantly, sty2-1 but not sty1-1 cells ectopically expressing the wis1 gene from the thiamine-repressible nmt1 promoter were able to proliferate at the restrictive temperature of 36.5°C [Fig. 2B]. We find that Δwis1 cells are also defective for cell division at 36.5°C [Table 2; data not shown]. To determine the relationship between sty2 and wis1, sty2-1 cells were crossed to Δwis1 cells. Analysis of >5000 spores from this cross revealed no wild-type recombinants, strongly suggesting that sty2 and wis1 are allelic. Supporting this argument a Δwis1 Δpyp1 Δpyp2 triple mutant strain was found to be viable [data not shown]. Because Δwis1 cells divide at an identical cell size to the sty2-1 allele, we conclude that sty2-1 harbors a null mutation in the wis1 MAP kinase kinase.

Sty1 encodes a MAP kinase homolog

We used the observation that sty1-1 cells are unable to proliferate at high temperature to isolate the sty1 gene. A genomic library was introduced into sty1-1 cells, and from a total of 50,000 transformants isolated at 30°C, 6 were able to divide at 36.5°C. Plasmids isolated from these six suppressors contain three different inserts that are related by restriction analysis and were subsequently shown to encode the same gene. pSTY1-4 containing the smallest insert is able to fully suppress the temperature sensitivity of a sty1-1 but not a sty2-1 mutant strain [Fig. 2C].

The restriction map of pSTY1-4 was not similar to a number of previously identified mitotic regulators, including cdc25 [Russell and Nurse 1986], wee1 [Russell and Nurse 1987a], nmt1 [Russell and Nurse 1987b], wis1 [Warbrick and Fantes 1991], cdc2 [Durkacz et al. 1986], or cdc13 [Fig. 3A; Hagan et al. 1988]. The complementing activity of pSTY1-4 was localized by transposon mutagenesis and subcloning analysis [Fig. 3B; Sedgewick and Morgan 1994]. Inactivating transposon insertions were used as templates for sequencing, which revealed a single open reading frame (ORF) of 1047 bp capable of encoding a polypeptide of 349 amino acids. The predicted protein sequence was analyzed against current data bases and found to be related most closely to a sub-family of MAP kinases that have been implicated in osmoregulation in budding yeast and stress-activated pathways in human cells [Brewster et al. 1993; Kyriakis et al. 1994, Han et al. 1994, Lee et al. 1994]. Specifically, the Sty1 protein kinase was found to be related most closely to HOG1 from budding yeast [86% identical] and 57% identical to CSB1 MAP kinase from human cells [Fig. 4A; Lee et al. 1994] and somewhat more distantly related [between 40% and 43% identical] to a family of four stress-activated protein kinases (SAPKs) from rat cells [Kyriakis et al. 1994]. All members of the MAP kinase superfamily so far described are activated by dual phosphorylation on two closely spaced residues that are located at residues Thr-171 and Tyr-173 in the Sty1 protein kinase was found to be related most closely to a sub-family of MAP kinases that have been implicated in osmoregulation in budding yeast and stress-activated pathways in human cells [Brewster et al. 1993; Kyriakis et al. 1994, Han et al. 1994, Lee et al. 1994]. Specifically, the Sty1 protein kinase was found to be related most closely to HOG1 from budding yeast [86% identical] and 57% identical to CSB1 MAP kinase from human cells [Fig. 4A; Lee et al. 1994] and somewhat more distantly related [between 40% and 43% identical] to a family of four stress-activated protein kinases (SAPKs) from rat cells [Kyriakis et al. 1994]. All members of the MAP kinase superfamily so far described are activated by dual phosphorylation on two closely spaced residues that are located at residues Thr-171 and Tyr-173 in the Sty1 pro-

Figure 2. Plasmid complementation of sty1 and sty2 mutants. [A] sty1-1 and sty2-1 mutants are temperature sensitive. Wild-type strain 972 [top left segment], sty1-1 cells [top right segment], and sty2-1 cells [bottom segment] were grown on YEPD and then streaked onto YEPD plates and allowed to grow for 3 days either at 30°C [left plate] or 36.5°C [right plate]. [B,C] Plasmid complementation of sty2-1 and sty1-1 mutants. sty2-1 ura4-D18 cells [B] or sty1-1 ura4-D18 cells [C] were transformed either with the control plasmid pUR19 [Cont.], pSTY1-4, a derivative of pUR19 containing 5.2 kb of genomic sequence pSTY1, or a plasmid expressing the wis1 ORF behind the thiamine-repressible nmt1 promoter pREP42-wis1 and the ura4+-selectable marker (pWIS1). Transformants were grown and streaked on minimal medium lacking thiamine and uracil and growth of the cells monitored after 3 days at either 30°C [left] or 36.5°C [right].
Table 2. Strains used in this study

| Strain no. | Genotype | Reference/source |
|------------|----------|------------------|
| PR 109     | leu1-32 ura4-D18 h+ | P. Russell (The Scripps Research Institute, San Diego, CA) |
| JM 1093    | leu1-32 ura4-D18 his1-102 h+ | this study |
| GL 193     | leu1-32 ura4-D18 ade6-704 h+ | this study |
| GL 196     | leu1-32 ura4-D18 cdc25-22 h+ | this study |
| PR 170     | leu1-32 ura4-D18 wee1-50 h+ | Russell and Nurse (1986) |
| GL 190     | leu1-32 ura4-D18 wee1-50 cdc25::ura4 h+ | Russell and Nurse (1986a) |
| PR 253     | leu1-32 ura4-D18 pypl::ura4 h+ | Millar et al. (1992b) |
| JM 1032    | leu1-32 ura4-D18 ade6-704 pypl2::LEU2 h+ | this study |
| JM 1023    | leu1-32 ura4-D18 NMT-pyp1(ura4) h+ | this study |
| JM 1036    | leu1-32 ura4-D18 ade6-704 NMT-pyp1(ura4) pypl2::LEU2 h+ | this study |
| JM 1042    | leu1-32 ura4-D18 his1-102 NMT-pyp1(ura4) pypl2::LEU2 h+ | this study |
| JM 1144    | leu1-32 ura4-D18 sty1-1 h+ | this study |
| JM 1162    | leu1-32 ura4-D18 sty2-1 h+ |this study |
| JM 1206    | leu1-32 ura4-D18 sty1-1 pyp1::ura4 pypl2::LEU2 | this study |
| JM 1207    | leu1-32 ura4-D18 sty2-1 pyp1::ura4 pypl2::LEU2 | this study |
| JM 1099    | leu1-32 ura4-D18 adh::pypl(ura4) pyp1+ h+ | this study |
| ED 890     | leu1-32 ura4-D18 ade6-M210 wis1::LEU2 h+ | Warbrick and Fantes (1991) |
| JM 544     | leu1-32 ura4-D18 wis1::ura4 h+ | this study |
| JM 1160    | leu1-32 ura4-D18 ade6-216 sty1::ura4 h+ | this study |

To determine the relationship between the sty1-1 mutation and the novel MAP kinase encoded by the pSTY1-4 genomic clone, the ORF of pSTY1-4 was interrupted by the ura4+ gene (Fig. 3C). The resulting plasmid pSTY1::ura4 was unable to rescue a sty1-1 mutant. pSTY1::ura4 was linearized with EcoRI and stably integrated into a leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M216/ade6-M210 h+/h- diploid strain. Tetrad dissection of asci from heterozygous diploids gave rise to four viable spores on germination that showed a 2:2 segregation of uracil auxotrophs to uracil prototrophs (data not shown).
Osmotic stress causes tyrosine phosphorylation of Sty1 and reduces cell size at division

The striking homology of Sty1 to the HOG1 MAP kinase from *S. cerevisiae* and of Wis1 to the HOG4 MAP kinase kinase prompted us to examine whether Δsty1 cells and Δwis1 cells were sensitive to osmotic stress. Neither Δsty1 nor Δwis1 mutants were able to proliferate in the presence of 1.5 M sorbitol or 0.8 M KCl (Fig. 5A), and arrested as highly elongated, branched, and septated cells [data not shown]. Wild-type cells continued to proliferate under these conditions [Fig. 5A]. These results confirm the previous observation that Δwis1 cells are sensitive to high salt conditions [Shiozaki and Russell 1995] and demonstrate that both sty1 and wis1 are critically required for the cellular response to osmotic stress in fission yeast.

We wished to examine whether Sty1 bears the hallmark of MAP kinases of being phosphorylated on tyrosine residues by the action of a MAP kinase kinase. Because initial attempts to identify the Sty1 protein in whole cell extracts were unsuccessful, an episomal plasmid ectopically expressing a carboxy-terminally tagged version of Sty1 from a partially defective thiamine-repressible nmt1 promoter was constructed. Log-phase cultures bearing this plasmid were subjected to osmotic stress and the Sty1 protein affinity precipitated. The phosphorylation state of Sty1 was assessed by Western blot using a monoclonal antibody to phosphotyrosine. Figure 5B demonstrates that in wild-type cells, the level of phosphotyrosine on the Sty1 protein increases rapidly after exposure to 0.9 M KCl. Duplicate samples probed using a monoclonal antibody to the hemagglutinin (HA) epitope tag showed that the level of protein did not change through the course of the experiment (Fig. 5B). In all experiments, phosphotyrosine was detectable on the Sty1 protein in vivo.

From our observation that wis1 and sty1 are required for the cellular response to osmotic stress, we hypothesized that cell size at division may be regulated by external osmolarity. To test this prediction, wild-type cells were grown to mid-log phase in rich medium either alone or in the presence of 1 M sorbitol and cell size at division measured. Although no difference in growth...
with the conclusion that the sty1 MAP kinase pathway controls cell size at division in response to osmotic stress. It was noted, however, that cell size at division of Δwis1 cells is reduced on rich medium as compared with minimal medium, as observed previously (Warbrick and Fantes 1991).

**Pyp2 MAP kinase phosphatase is induced by osmotic stress**

The role of ppy1 and ppy2 in the regulation of the sty1 MAP kinase pathway was examined. Overexpression of ppy1 was found to abolish the increase in phosphotyrosine content on the Sty1 protein induced by osmotic stress [Fig. 5B], raising the possibility that Sty1 may be a direct substrate for Pyp1 and Pyp2 PTPases. Initial experiments showed that neither the ppy1 nor ppy2 mRNAs are periodically expressed in the cell cycle [data not shown]. However, we observed that the ppy2 mRNA, although low in normal medium, increases dramatically following exposure to 0.9 M KCl, reaching a maximum after ~1 hr and declining to basal levels by 3 hr [Fig. 6A]. In contrast, neither cdc2 mRNA [Fig. 6A] nor ppy1 mRNA was altered during the time course of this experiment [data not shown]. Similar results were obtained when cells were exposed to medium containing 1.2 M sorbitol [data not shown]. Importantly, no induction of ppy2 mRNA was observed in a Δwis1 strain [Fig. 6A] or in a sty1-1 mutant (M. Wilkinson and J. Millar, unpubl.), indicating that the sty1 MAP kinase pathway is required for induction of ppy2 mRNA in response to osmotic stress.

These results prompted us to examine whether the tyrosine phosphorylation state of Sty1 was influenced by ppy2 on prolonged exposure to osmotic stress. In a wild-type strain, the Sty1 protein was found to be maximally phosphorylated between 15 and 30 min after exposure to 0.9 M KCl and declined to basal levels after ~1 hr [Fig. 6B]. Notably, the time course of tyrosine dephosphorylation coincides well with the time course of ppy2 mRNA induction [Fig. 6A]. To examine whether this dephosphorylation was influenced by ppy2, epitope-tagged Sty1 was introduced into a Δppy2 strain. In these cells the percentage increase in phosphotyrosine on the Sty1 protein was only 68% in three independent experiments after 15 min of incubation with 0.9 M KCl compared to 485% observed in a wild-type strain. In contrast to wild type, Sty1 remained tyrosine phosphorylated on prolonged exposure to osmotic stress in Δppy2 cells [Fig. 6B]. Little difference in the time course of phosphorylation and dephosphorylation was observed in a Δppy1 strain relative to wild type [data not shown]. These results suggest that Ppy1 and Ppy2 perform distinct roles in the tyrosine dephosphorylation of Sty1 and that Ppy2 acts in a feedback pathway to tyrosine dephosphorylate Sty1.

To determine whether the Ppy2 protein could directly influence the phosphorylation state of Sty1 in vitro, tyrosine-phosphorylated Sty1 was incubated in the presence of a glutathione S-transferase (GST)–Ppy2 fusion
Dephosphorylation by GST–Pyp2 was greatly inhibited by vanadate, a general PTPase inhibitor (Fig. 6C). During these experiments we noted that the Pyp2 PTPase forms a weak but specific association with Styl. Lysates containing epitope-tagged Styl were incubated in the presence of either GST–Pyp2 or an equivalent concentration of a functionally unrelated PTPase fusion protein, GST–Pyp3 (Millar et al. 1992a). The results in Figure 6D illustrate that epitope-tagged Styl specifically coprecipitates with GST–Pyp2, whereas no association with GST–Pyp3 was observed. Together, these data indicate that Pyp2 is a MAP kinase phosphatase that acts in a feedback loop to inactivate Styl following exposure to osmotic stress [Fig. 7].

**Discussion**

Protein modification by tyrosine phosphorylation, although relatively common in the control of proliferation, differentiation, and intercellular communication between mammalian cells, is rare in yeast. Only recently has the discovery of a number of tyrosine phosphatases in fission yeast revealed an important role for tyrosine phosphorylation in cellular growth control of unicellular eucaryotes. This study was initiated to uncover the mechanism of action of two tyrosine-specific phosphoprotein containing the carboxy-terminal catalytic 303 amino acids of the Pyp2 protein [Millar et al. 1992b]. As the results in Figure 6C demonstrate, GST–Pyp2 was able to reduce the level of phosphoryrosine on Styl by ~90%. Dephosphorylation by GST–Pyp2 was greatly inhibited by vanadate, a general PTPase inhibitor [Fig. 6C]. During these experiments we noted that the Pyp2 PTPase forms a weak but specific association with Styl. Lysates containing epitope-tagged Styl were incubated in the presence of either GST–Pyp2 or an equivalent concentration of a functionally unrelated PTPase fusion protein, GST–Pyp3 (Millar et al. 1992a). The results in Figure 6D illustrate that epitope-tagged Styl specifically coprecipitates with GST–Pyp2, whereas no association with GST–Pyp3 was observed. Together, these data indicate that Pyp2 is a MAP kinase phosphatase that acts in a feedback loop to inactivate Styl following exposure to osmotic stress [Fig. 7].

**Figure 6.** Pyp2 is a MAP kinase phosphatase. (A) Pyp2 mRNA is induced by osmotic stress. Log-phase cultures of either wild-type [WT] or Δwis1 cells were incubated in YEPD containing 0.9 M KCl for the times indicated. Total RNA was extracted and probed using DNA specific to the pyp2 or cdc2 genes. (B) Effect of pyp2 on tyrosine phosphorylation of Styl. Log-phase cultures of leu1-32 ura4-D18 [WT], or pyp2::ura4 leu1-32 ura4-D18 [ΔPyp2] cells transformed with pREP41-sty1[HA-6His] were incubated in 0.9 M KCl for the times indicated. Styl was isolated by Ni²⁺-NTA affinity precipitation and probed by Western blot for the presence of phosphotyrosine (α-PTyr) or HA epitope tag (α-HA). (C) Pyp2 PTPase dephosphorylates Styl in vitro. Tyrosine-phosphorylated Styl protein was isolated by affinity precipitation from osmotically stressed wild-type cells expressing epitope-tagged Styl. Beads were incubated either in the presence (+) or absence (−) of 2 mM orthovanadate and either no protein [Control] or 10 μg of GST–Pyp2 fusion protein or 10 μg of GST–Pyp3 fusion protein for 30 min at 30°C. Styl was then probed by Western blot for the presence of phosphotyrosine [α-PTyr] or HA epitope tag [α-HA]. (D) Pyp2 associates with the Styl MAP kinase. Cell lysates containing epitope-tagged Styl were incubated in the presence of no protein [Control], 10 μg of GST–Pyp2 fusion protein, or 10 μg of GST–Pyp3 fusion protein for 1 hr at 4°C. After this time proteins were precipitated on glutathione-agarose beads, washed three times with lysis buffer, and probed for the presence of Styl using a monoclonal antibody to the HA epitope tag.

**Figure 7.** Osmotic control in fission yeast. This model depicts the genetic and biochemical interactions described in this paper. Osmotic stress induces activation of Wis1 by an as-yet-unidentified MAP kinase kinase kinase [or MEKK]. This leads to dual phosphorylation of Styl on residues Thr-171 and Tyr-173 to induce its activation. Activation of Styl leads to an advancement in the timing of mitosis and a reduction in cell size at division. We propose a feedback loop by which activation of Styl leads to accumulation of the pyp2 mRNA presumably through the action of an unidentified transcription factor, which in turn leads to dephosphorylation of Styl and its inactivation. The phosphatases that dephosphorylate Thr-171 on the Styl protein have not been identified.
phatases, pyp1 and pyp2, shown previously to act as inhibitors of mitotic initiation in fission yeast and to share an essential overlapping function (Millar et al. 1992b; Ottolie et al. 1992). We examined the mechanism underlying this loss of viability by isolating spontaneous mutations that could bypass the requirement for pyp1 and pyp2.

**Sty1 and Wis1 are required for the cellular response to osmotic stress**

We have isolated mutations in two complementation groups that define a MAP kinase signaling pathway controlling cell size at division in fission yeast. We have identified the central members of this pathway as the Wis1 MAP kinase kinase and Sty1 MAP kinase. We present evidence that the Pypl and Pyp2 PTPases function in opposition to the Wis1 MAP kinase kinase to control tyrosine phosphorylation and, presumably, activity of the Sty1 MAP kinase. Sequence analysis reveals that Sty1 is closely related to a subfamily of MAP kinases that have been implicated in osmoregulation in both budding yeast and mammalian cells (Brewster et al. 1993; Han et al. 1994; Lee et al. 1994). We demonstrate that the sty1 and wis1 genes are themselves required for osmoregulation in fission yeast by virtue of the observations that, first, cells bearing deletions of either gene are sensitive to a number of independent osmotic stresses, and second, Sty1 is tyrosine phosphorylated following osmotic stress in a manner dependent on the wis1 MAP kinase kinase. Thus, Sty1 is a structural and functional homolog of budding yeast HOG1, and Wis1 is the MAP kinase kinase that tyrosine phosphorylates and activates Sty1 in vivo. Because no prior assumptions were made as to the function of pyp1 and pyp2, we note that it was fortuitous that the suppression screen in this study was performed in the presence of 1 M sorbitol in which sty1- and wis1- mutants are viable, and not in the presence of 1.5 M sorbitol, conditions under which they are unable to continue proliferating.

**Loss of pyp1 and pyp2 causes hyperactivation of Sty1**

Simultaneous inactivation of pyp1 and pyp2 causes aberrant cellular morphology and growth arrest. Several lines of evidence suggest that this is because of hyperactivation of the Sty1 MAP kinase. First, the synthetic lethality of a Δpyp1 Δpyp2 double mutant is suppressed by a mutation in either the sty1 or wis1 genes. Second, strong overexpression of the wis1 MAP kinase kinase from the thiamine-repressible nmt1 promoter is lethal and exhibits a terminal phenotype indistinguishable from the conditional NMT-pyp1 Δpyp2 cells, an effect that is not observed in a sty1-1 mutant or in a strain constitutively overexpressing the pyp1 PTPase (J.-C. Shieh and J.B.A. Millar, unpubl.). The mechanism by which hyperactivation of the Sty1 kinase causes a loss of normal cell morphology and growth arrest is uncertain at present. Hyperactivation of Sty1 may lead to a lethal increase in intracellular osmolarity such that cells eventually die by lysis. Alternatively, the Sty1 MAP kinase pathway may directly control cell polarity. We are attempting to distinguish between these possibilities. Recent evidence that correct positioning of the actin cytoskeleton in *S. cerevisiae* after osmotic stress requires the activity of the HOG1 MAP kinase supports the latter model (Brewster and Gustin 1994). Intriguingly, activation of a close homolog of Sty1 in rat PC12 cells leads to phosphorylation of Hsp27, a small heat shock protein implicated in actin filament stability (Lavoie et al. 1993; Rouse et al. 1994).

**Distinct roles for the Pypl and Pyp2 MAP kinase phosphatases**

The genetic data in this paper strongly suggest that both the Pypl and Pyp2 PTPases inhibit the sty1 MAP kinase pathway. This conclusion is supported by the observation that both the Pypl and Pyp2 PTPase influence the tyrosine phosphorylation state of Sty1 in vivo and that the Pyp2 PTPase associates with and tyrosine dephosphorylates Sty1 in vitro. In particular, we observe that the dephosphorylation of Sty1 on prolonged exposure to osmotic stress is partially defective in a Δpyp2 strain. We find that the pyp2 mRNA is induced strongly under these conditions, indicating that Pyp2 acts in a feedback loop to dephosphorylate Sty1. Notably, the mRNAs for a number of dual-specificity MAP kinase phosphatases are induced by extracellular stimulation, including human MKP1, CL100, PAC1, and budding yeast MSG5, although the mechanism of induction is not known (Keyse and Emslie 1992; Sun et al. 1993; Doi et al. 1994; Ward et al. 1994). We demonstrate that both wis1 and sty1 are required for induction of pyp2 mRNA, presumably via the action of an as-yet-unidentified transcription factor. Notably, some dephosphorylation of Sty1 was observed consistently in a Δpyp2 strain indicating the existence for other mechanisms of Sty1 inactivation. In contrast to pyp2, the pyp1 mRNA is not induced by osmotic stress indicating that Pypl and Pyp2 PTPases perform distinct roles as MAP kinase phosphatases for Sty1. The precise role of Pypl in Sty1 dephosphorylation is under further investigation.

Because Pypl and Pyp2 PTPases are specific for tyrosine residues, these data also suggest that two distinct classes of MAP kinase phosphatase act to dephosphorylate residues Thr-171 and Tyr-173 in the Sty1 protein. Pertinent to this, mutations that bypass the lethal combination of the deletion of two type 2C phosphatases ptc1 and ptc3 in fission yeast have been found to reside in the wis1 MAP kinase kinase and a second locus, spc1 [Shiozaki and Russell 1995]. Sequence comparison revealed that spc1 and sty1 are the same gene, indicating a role for type 2C serine/threonine phosphatases in the inactivation of this pathway [K. Shiozaki and P. Russell, pers. comm.]. Similarly, Maeda and colleagues have demonstrated that overexpression of a tyrosine-specific phosphatase, PTP2, or two serine/threonine-specific type 2C phosphatases, PTC1 and PTC3, reverse
hyperactivation of the HOG1 MAP kinase pathway caused by inactivation of the SLN1 gene [see below]; however the targets of these phosphatases have not been established [Maeda et al. 1994]. Further experimentation will be required to determine the nature of the phosphatases regulating Thr-173 phosphorylation on the Sty1 protein.

An osmosensing MAP kinase pathway controlling cell size at division

The ppy1 and ppy2 PTPases have been implicated in the control of mitotic initiation in fission yeast on the basis of their effects on cell size at division [Millar et al. 1992b; Orlilie et al. 1992]. In this study we demonstrate that ppy1 and ppy2 mediate control of cell size at division by dephosphorylating Sty1 and act in opposition to the wis1 MAP kinase kinase. Notably the wis1 gene was initially described as a dose-dependent initiator of mitosis [Warbrick and Fant1 1991]. We show that loss of sty1 delays the timing of mitotic initiation. We demonstrate further that wis1 and sty1 are required for the cellular response to osmotic stress and that chronic exposure to osmotic stress causes a reduction in cell size at division. These results strongly support the conclusion that the sty1 MAP kinase pathway regulates cell size at division in response to changes in external osmolarity, thus revealing a potentially vital link between the detection of environmental stress and control of the cell cycle.

Physiological and genetic studies indicate that the initiation of mitosis in fission yeast requires attainment of a critical cell mass [Nurse 1975, Fant1 and Nurse 1977]. Thus, mutations that alter the timing of mitosis uncouple size control from cell cycle progression. In principle, the sty1 MAP kinase pathway may also alter mass control over the initiation of mitosis by impinging on the activity of the cdc2 kinase. An alternative explanation is that osmotic stress and activation of the sty1 MAP kinase pathway do not alter cell size control governing the initiation of mitosis but lead to an increase in intracellular density, for example, by an increase in intracellular glycerol concentration. To maintain mass control over the initiation of mitosis, cells undergo a reduction in cell volume. Because S. pombe cells grow by length extension this would be expressed as a reduction in cell size at division. Further experimentation will be required to determine which of these hypotheses is correct and to elucidate the molecular basis underlying this response. It has been known for some time that exposure of human cells to anisotonic media initiates a response that regulates cellular volume (Grinstein and Foskett 1990). Furthermore, it is now recognized that osmotic stress activates a number of MAP kinases including JNK1 and p38 in mammalian cells that share extensive sequence homology to Sty1 [Galcheva-Gargova et al. 1994; Han et al. 1994], raising the possibility that the mechanisms controlling eucaryotic cell size may be evolutionarily conserved.

A role for the Sty1 MAP kinase pathway in stationary phase!

Previous data have demonstrated that mutants in the wis1 MAP kinase rapidly lose viability in stationary phase suggesting that this pathway is influenced by nutritional stimuli [Warbrick and Fant1 1991]. Cells overexpressing the ppy1 PTPase and Δsty1 cells exhibit the same phenotype, indicating that the sty1 MAP kinase may be activated on exit from the cell cycle [J.B.A. Millar, unpubl.]. The nature of the signal that might activate the pathway upon entry into stationary phase is at present unclear, though we believe this is unlikely to be attributable to changes in external osmolarity of the medium. Unlike sty1 and wis1, no defect in cell size at division, long-term survival in stationary phase or sensitivity to growth at high temperature has been ascribed to budding yeast bearing mutations in the HOG1 and HO44 gene [Brewster et al. 1993]. Furthermore, wis1 does not complement a HO44 mutation nor vice versa, suggesting a possible evolutionary divergence between these two yeasts. It is intriguing that the mouse p38 MAP kinase is activated not only by osmotic stress but also by ultraviolet light, heat shock, bacterial endotoxic lipopolysaccharide [LPS], and stress related pro-inflammatory cytokines such as tumor necrosis factor α [TNFα] and interleukin-1 [IL-1] [Freshney et al. 1994; Han et al. 1994; Rouse et al. 1994; Dérijard et al. 1995], demonstrating that more than one stimulus can modulate the activity of this pathway in metazoans. It will be of great interest to determine the range of extracellular signals, other than osmotic stress, that control the sty1 MAP kinase pathway in fission yeast and to what extent this pathway is evolutionarily conserved.

The activity of MAP kinase kinases is stimulated via phosphorylation by a MAP kinase kinase kinase (for review, see Herskowitz 1995; Marshall 1994). In the genetic suppression screen performed in this study, only two complementation groups were identified that were viable in the absence of thiamine. This may indicate a functional redundancy in the action of upstream regulators of wis1 or that mutations in such genes are lethal. Notably a set of mutants, which were lethal in the absence of thiamine, were isolated in the same screen and may include mutants in such upstream regulators. These are now the subject of further investigation. In budding yeast, a two-component system consisting of the SLN1 and SK1 genes has been shown to regulate the HOG1 MAP kinase [Maeda et al. 1994], raising the exciting possibility that a similar system may control the sty1 MAP kinase in fission yeast and possibly higher eukaryotes.

In conclusion, we have identified a conserved MAP kinase pathway in fission yeast that coordinates the cellular response to environmental stress with control of cell size at division. Recently, it has become apparent that stress-activated MAP kinase pathways may play an important role in a variety of physiological and pathological responses in mammals, including inflammation and tumor suppression. The closest human homolog to Sty1, CSBP1, has been shown to be the target of a group of
synthetic drugs that not only suppress accumulation of the stress related pro-inflammatory cytokines, TNFa and IL-1, but also act as potent therapeutic agents in a number of animal models of chronic and acute inflammation [Lee et al. 1994]. We contend that further genetic dissection of the sty1 MAP kinase pathway will advance our understanding of the mechanisms governing the cellular response to environmental stress, and provide an ideal model system with which to study these novel MAP kinase pathways in human cells.

**Materials and methods**

**Media and general techniques**

Media and genetic methods for studying fission yeast have been reviewed recently [Moreno et al. 1991]. General DNA methods were performed using standard techniques [Sambrook et al. 1989]. Cell length measurements were made using log-phase cells with a Nikonfilar eyepiece drum micrometer at 1200× magnification. Transformations were regularly performed by lithium acetate method [Moreno et al. 1991] or by electroporation [Prentice 1991] using a Bio-Rad Gene Pulser.

**Construction of NMT–pypl(ura4) and pyp2::LEU2 alleles**

The ppy1 ORF was cloned by PCR amplification from an S. pombe cDNA library. The 5′ oligonucleotide, CCGGATC-CATATGAAATTTTCAAACCGTTAACAA, incorporating BamHI and Ndel sites (shown italicized) hybridized to sequences surrounding the ATG initiation codon, whereas the 3′ oligonucleotide, CCGGATCCTGATCTTTAAACCCGGAAATGAC, incorporating a BamHI site (shown italicized) hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 1.7-kb fragment that was cleaved with Ndel and BamHI and cloned into the Ndel and BamHI sites of pREP82 [Maundrell 1993] containing the thiamine-repressible nmt1 promoter to form pREP82–ppy1. A 1.2-kb fragment containing the ars1 autonomously replicating element was excised by digestion with EcoRI followed by self-ligation. The carboxyterminal half of ppy1 containing the PTase catalytic domain was removed by digestion with ScaI to drop out a 692-bp fragment followed by self-ligation to form pRIP82–ppy1(T). The NMT–ppy1 strain was created by linearizing pRIP82–ppy1(T) using the unique SalI site in ppy1 and introduced into a leu1–32 ura4–D18 h+ strain by lithium acetate transformation onto minimal medium lacking uracil and genomic DNA subjected to Southern blot hybridization. The resulting haploid uracil prototrophs were identified and contained overlapping sequences, pSTY1–1 and cloned into the NotI and Ndel sites of pREP82 [Maundrell 1993] containing the thiamine-repressible nmt1 promoter to form pREP82–ppy1. A 1.2-kb fragment containing the ars1 autonomously replicating element was excised by digestion with EcoRI followed by self-ligation. The carboxyterminal half of ppy1 containing the PTase catalytic domain was removed by digestion with ScaI to drop out a 692-bp fragment followed by self-ligation to form pRIP82–ppy1(T). The NMT–ppy1 strain was created by linearizing pRIP82–ppy1(T) using the unique SalI site in ppy1 and introduced into a leu1–32 ura4–D18 h+ strain by lithium acetate transformation onto minimal medium lacking uracil. Stable integrants were identified and verified by Southern blot hybridization.

To create the ppy2::LEU2 allele a 5.1-kb fragment from pGEM-3Z–ppy2 [Millar et al. 1992b] was excised with EcoRI and ligated into the EcoRI site of pBluescript to form pBSKK–ppy2. A 2.2-kb fragment containing LEU2 was excised from pREP1 with HindIII, blunt ended with the DNA polymerase I, and ligated into pBSKK–ppy2 that had been digested with HpaI and NraI to excise a 1515-bp fragment containing much of the ppy2 ORF to form pBSKK–ppy2::LEU2. The disrupted ppy2 sequence was excised from pBSKK–ppy2::LEU2 with PstI and HindIII and introduced into a leu1–32 ura4–D18 ade6–704 h+ strain using lithium acetate transformation onto minimal media plates lacking leucine. Stable integrants were isolated and confirmed by Southern blot hybridization.

**Isolation of spontaneous suppressors of NMT–ppy1**

Two strains, JM1036 and JM1042, bearing the NMT–ppy1 Δppy2 alleles were grown to stationary phase in minimal me-
mined after at least 48 hr growth in the absence of thiamine. The pREP41–styl1[HA–6His] plasmid was found to rescue the temperature sensitivity of a styl-1 leu1-32 ura4–D18 strain when grown in the absence of thiamine.

Detection of Sty1 protein

The Sty1 protein was partially purified from cells containing the pREP41–styl1[HA–6His] plasmid (see above), which expresses a fusion protein of Sty1 to a HA peptide epitope and a six histidine carboxy-terminal tail. Briefly, pelleted cells were lysed into lysis buffer (0.5% NP-40, 0.5% Na-deoxycholate, 50 mM NaF, 10% glycerol, 2 mM Na-orthovancadate, 10 mM β-mercaptoethanol, 10 μg/ml of aprotinin, 10 μg/ml of benzamidine, 2 mM PMSF, 10 μg/ml of pepstatin A, 10 μg/ml of leupeptin, 50 mM Tris-HCl at pH 7.4) and the Sty1 protein isolated by affinity precipitation on Ni2+–NTA beads (Qiagen). Precipitated proteins were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with either a monoclonal antibody to the HA epitope (12CA5) or with monoclonal antibody to phosphotyrosine (4G10, UBI). Detection was performed using peroxidase-conjugated anti-mouse IgGs (Amersham, UK) and chemiluminescence visualization (ECL, Amersham) according to the manufacturer’s instructions.

Phosphatase assay

GST–Ppy2 and GST–Ppy3 fusion proteins were made in Escherichia coli as described previously [Millar et al. 1992a,b]. Phosphatase assays were performed in phosphatase buffer (1 mM EDTA, 2 mM DTT, 200 mM imidazole at pH 7.2) as described previously [Millar et al. 1991].

Construction of wis1::ura4 allele

The wis1 gene was cloned by PCR amplification from an S. pombe cDNA library. The 5’ oligonucleotide, CCCCTCGAG-GATATGGTCTGATCTTCTCTCACAATACAAACC, incorporating Xhol, NdeI, XbaI, and BglII sites [shown italicized], hybridized to sequences surrounding the ATG initiation codon, whereas the 3’ oligonucleotide, CACTGCAGCGGCCGCT-TCTTCTCCAAATAATCAACCC, incorporated SstI, XhoI, and BglII sites (shown italicized), hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 1846-nucleotide fragment that was cleaved with PstI and XhoI and cloned into the PstI and XhoI sites in pBluescript to form pBSSK–wis1. This plasmid was cleaved with HindIII, and a 1.66-kb HindIII fragment containing the S. pombe ura4* gene was ligated into this site to form pBSSK–wis1::ura4. Plasmid pBSSK–wis1::ura4 was cleaved with PstI and XhoI and used to transform a leu1-32 ura4–D18 h− strain. Stable uracil prototrophs were selected. Confirmation of the wis1::ura4 disruption was determined by PCR amplification using the oligonucleotides described above.

Overexpression of wis1

The wis1 ORF was amplified by PCR using the oligonucleotides described above and cleaved with NdeI and NotI and ligated into the NdeI and NotI sites of pREP42 [HA–6His] [J. Millar, unpubl.] to form pREP42–wis1 [HA–6His]. This plasmid was used to transform strains bearing the ura4–D18 mutation and uracil prototrophs selected. The phenotype of overexpression was determined after at least 48 hr growth in the absence of thiamine.

DNA and RNA isolation and hybridization

S. pombe cells were cultured in YEA medium (0.5% yeast extract, 3% glucose, 50 mg/liter of adenine) to stationary phase. Chromosomal DNA isolated from a 10-ml culture was dissolved in 25 ml of TE, of which one-fifth was digested with HindIII and subjected to electrophoresis and Southern blot hybridization. A BglII fragment isolated from the pSTY1-4 gene containing sty1 sequence was radioactively labeled and used as a probe. To isolate RNA, S. pombe cells were cultured in YEPD to exponentially growing phase. Approximately 10 μg of total RNA was isolated and resolved by agarose gel electrophoresis and transferred to nitrocellulose for hybridization as described previously [Aves et al. 1985]. A 1-kb EcoRV fragment from pGEM–3z–ppyp2 was used to probe for ppyp2 mRNA. A 1-kb Ndel–BamHI fragment from pREP1–cdc2 [HA–6His] was used to probe for cdc2 mRNA.

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