**PAT1**, an evolutionarily conserved acetyltransferase homologue, is required for multiple steps in the cell cycle

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Communicated by: Virginia Zakian

Abstract

Background: Acetylation has been implicated in many biological processes. Mutations in N-terminal acetyltransferases have been shown to cause a variety of phenotypes in *Saccharomyces cerevisiae* including activation of heterochromatin, inability to enter G0, and lethality. Histone acetylation has been shown to play a role in transcription regulation, histone deposition and histone displacement during spermatogenesis, although no known histone acetyltransferase is essential.

Results: Studies aimed at revealing a role for histone H1 in yeast have uncovered a mutation in a putative acetyltransferase, *PAT1*. The mutant (*pat1-1*) cells can live only in the presence of vertebrate H1. *PAT1* is essential for mitotic growth in *S. cerevisiae*; mutant cells depleted of the Pat1p show aberrant cellular and nuclear morphology. *PAT1* is required for multiple cell cycle events, including passage through START, DNA synthesis, and proper mitosis through a microtubule-mediated process. The *S. pombe PAT1* gene was cloned by complementation and shown to exist as part of a larger protein, the unique portion of which is homologous to a second *S. cerevisiae* gene. *pat1* mutants show a variety of mitotic defects including enhanced chromosome loss, accumulation of multiple nuclei, generation of giant cells, and displays classical cut phenotypes in which cytokinesis occurs in the absence of proper nuclear division and segregation.

Conclusion: *PAT1* controls multiple processes in cell cycle progression which suggests an essential role for the acetylation of yet unknown substrate(s).

Introduction

The primary structural unit of chromatin in eukaryotes is the nucleosome, which consists of two copies of each core histone and 146 bp of DNA (McGhee & Felsenfeld 1980). Higher eukaryotes also contain an additional histone, H1, thought to be involved in higher order chromatin structure and possibly transcriptional control (Allan et al. 1981; Zlatanova 1990). All core histones can be divided into two functional domains: a hydrophobic carboxy-terminal domain and a hydrophilic amino-terminal tail (Smith 1991; Wolffe & Pruss 1996a). The hydrophobic domains of core histones are required for nucleosome assembly and cell viability. Deletions of the N-terminal domain of any of the core histones are viable (Wallis et al. 1983; Schuster et al. 1986; Kayne et al. 1988). However, deletion of the N-terminal domain of histone H4 de-represses the silent mating site locus in yeast, suggesting that this domain might play a role in the formation of heterochromatin in vivo (Kayne et al. 1988). The N-terminal domain of histone H4 contains several highly conserved lysines that are differentially subjected to reversible acetylation. Acetylation neutralizes the positive charges on lysines and is thought to regulate its interaction with negatively charged DNA (Allfrey 1977). Mutational analysis suggests that the ability to
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**Diagram (A):**

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**Diagram (B):**

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ATG

pRL57/pRL58

pRL57 (pat1-1-Δ2::His3)

His3+KAN

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**Diagram (C):**

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Pat1p

SPat1p

STAPH

STAT

SSAT

AAC

Mak3p

Scs554Dm

Hap1p

Sas2p

Ybl052C

Arclp

Gcn5p

Npl2p

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modulate the positive charges on H4 amino terminus by acetylation is more important than the positive charge at these highly conserved positions (Megee et al. 1990; Park & Szostak 1990; Durrin et al. 1991).

Acetylation is an energy intensive, dynamic phenomenon whose steady-state balance is mediated by the opposing activities of histone acetyltransferase and deacetylase enzyme systems. Recently, the non-essential yeast gene GCN5 (Georgakopoulou & Thireos 1992), was shown to be an A-type histone acetyltransferase, demonstrating a strong link between histone acetylation and regulated transcription (Brownell et al. 1996; Wollfe & Pruss 1996b), and a mammalian histone deacetylase has been related to the yeast transcriptional regulator Rpd3p (Taunton et al. 1996). Taken together, these data indicate that the steady-state balance of histone acetylation plays a direct role in the modulation of chromatin structure to create new patterns of transcription (Brownell & Alliss 1996).

Histone H1, unlike core histones, is highly diverged in sequence among species. However, most histone H1s have a high percentage of lysines and share a distinctive secondary structure: a central globular domain that is flanked by a short N-terminal tail and a long, highly charged C-terminal tail (Allan et al. 1980). Although histone H1 has been implicated in chromatin compaction and gene regulation (Allan et al. 1981; Zlatanova 1990), its role in vivo in genetically tractable eukaryotes such as S. cerevisiae is still unclear. This is in part due to the fact that H1 has not been identified in genetically tractable eukaryotes such as S. cerevisiae, although a number of conventional approaches have been taken (Certa et al. 1984; Srebreva et al. 1987).

Electron microscopic analyses have indicated that chromatin in yeast cells can fold into a higher-order structure that appears similar to the 30-nm filaments of higher eukaryotic cells, suggesting that histone H1 or H1-like protein exists in yeast (Allan et al. 1984; Lowary & Widom 1989).

In this study, we describe an attempt to identify a yeast histone H1 homologue by screening for yeast mutants whose growth is dependent on the expression of a vertebrate H1. Consistent with the results of Linder & Thoma (1994), we found that the low level expression of an exogenous histone H1 in yeast does not cause any obvious phenotype. A mutant that appears to be dependent on H1 expression was isolated by the colony-colour-sectoring assay. The wild-type gene responsible for this phenotype was cloned and characterized. It encodes a small novel protein whose function is essential for mitotic growth in Saccharomyces cerevisiae and that shares significant homology to acetyltransferases. Genetic analyses have indicated that the PAT1 gene is involved in multiple steps in the cell cycle: passage through START after an α-factor block, the progression of DNA synthesis, and mitosis through a microtubule-mediated process.

Results
Isolation of histone H1-dependent mutants

The chicken H1 11L (chH1) genomic sequence was amplified by PCR and cloned into a centromeric expression vector pMW29. The resulting plasmid, pMW29H1, contains a GAL1-chH1 fusion. pMW29H1 was transformed into YMW1-6 and its effect on cell growth was analysed. This strain has doubling times of 81 min in SC glucose and 124 min in SC galactose media at 30 °C, which are identical to the doubling times of YMW1-6 under the same conditions. Expression of chH1 was confirmed by both mRNA

Figure 1  (A) Deletion analysis defines the PAT1 genomic locus. A deletion series was generated from a complementing genomic clone and tested for their ability to complement the pat1-1 mutation; plus signs (+) indicate clones that complement and minus signs (−) indicate a failure to complement. The complementation activity was narrowed to a 1.5 kb SaI fragment whose sequence is shown in (B). The closed arrowhead points to the first ATG and the open arrow indicates the termination codon. pat1-Δ2::HIS3 is a disrupted allele in which Not-Ball fragment is replaced by a HIS3 cassette indicated as a hatched box. (B) The nucleotide sequence of the 1.5 kb SaII fragment and predicted amino acid sequence of Pat1p. The numbering for both nucleic acid (plain) and amino acid (bold) are shown to the left of these sequences. The region between amino acid 97 and 148 which is referred to as the ‘acetyltransferase domain’ in (C) is shaded. (C) Comparison of the Pat1p sequence with other acetyltransferases and related sequences, including Staphylococcus aureus atl gene product (STAPH, GENBANK accession number D17366), Escherichia coli streptomycin acetyltransferase (Sat3, g669115), Pseudomonas aeruginosa 6’-N-acetyltransferase (AAC, M29695), Mouse spermidine/spermine N1-acetyltransferase (SSAT, L10244), Saccharomyces cerevisiae L-A virus protein N-acetyltransferase (MAK3, Q03503), Saccharomyces cerevisiae hypothetical protein (SC8554.04, Z46796), Saccharomyces cerevisiae protein involved in silencing HMR (Sac2p, U15458), Saccharomyces cerevisiae hypothetical protein (YBL052C, Z35814), Saccharomyces cerevisiae N-terminal acetyltransferase (Arp1p, M11621), Saccharomyces cerevisiae histone acetyltransferase (Hat1p, U33335), and Saccharomyces cerevisiae transcriptional activator (Gcn5p, X68628). Gaps are allowed to generate the best alignment. Identical residues (to Pat1p) are indicated in black whereas conserved residues are shaded in grey.

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A genetic screen was performed to identify mutants whose growth is dependent on the expression of \( chH1 \). These mutants are expected to be defective either in the yeast histone H1 homologue or other genes that are suppressible by chicken H1 protein. Cells with lethal mutations in the desired gene are unable to grow in the absence of the plasmid carrying \( chH1 \) and can be identified by screening for mutants that are unable to lose pMW29H1 in the absence of nutritional selection. The screen is based on the colony-colour-sectoring assay described previously (Kranz & Holm 1990; Zieler et al. 1995) with which the plasmid loss can be easily visualized by a colour change. This assay took advantage of the fact that ade2 yeast cells accumulate red pigments but ade2 ade3 cells do not. When an ade2 ade3 strain carries \( ADE3 \) on an autonomously replicating plasmid, it forms red colonies. White sectors form in red colonies when cells lose plasmids during colony growth (referred to as Sect\(^-\) phenotype).

Three main criteria were anticipated for H1-dependent mutants; failure to survive plasmid loss resulting in homogeneously red, non-sectoring (Sect\(^+\)) colonies; inability to grow on plates containing 5-fluoro orotic acid (5-FOA), and failure to grow when \( chH1 \) expression is repressed on glucose media. To isolate H1-dependent mutants, YMW1-6 containing pMW29H1 was mutagenized and plated on YPG (galactose) plates. Forty Sect\(^-\) mutants were isolated from 30,000 examined colonies. These mutants were tested for their ability to grow on SC-uracil media and sensitivity to 5-FOA to ensure that the Sect\(^-\) phenotype was due to the retention of pMW29H1 rather than reasons unrelated to the plasmid. Yeast cells that contain \( URA3 \) are sensitive to 5-FOA. Among the 40 Sect\(^-\) mutants, 11 were able to grow on SC-uracil plates but not 5-FOA plates. These 11 mutants were further tested for their dependency on galactose, which is indicative of the dependency on the \( chH1 \) expression. The \( GAL1 \) promoter is repressed in the presence of glucose but activated in the absence of glucose and in the presence of galactose. Only one mutant, YRL4, showed lethality on YPD plates. The other 10 Sect\(^-\) mutants that showed 5-FOA sensitivity but not galactose dependency were similar to the background mutants observed in Kranz & Holm (1990). The reason most of these mutants maintain pMW29H1 was a requirement for either \( ADE3 \) or \( URA3 \) genes. Using a plasmid shuffle protocol (Rose & Fink 1987), we were able to show that these mutants can lose pMW29H1 when a wild-type copy of \( URA3 \) or \( ADE3 \) on a second plasmid was introduced (data not shown). They are likely to be mutations involved in nucleoside metabolic pathways.

YRL4 was the only mutant that met all three criteria: Sect\(^-\), 5-FOA sensitivity, and galactose-dependency. The galactose dependency suggests that the mutation in YRL4 is suppressed by the expression of H1 and not another gene on the plasmid. However, the phenotype of YRL4 appears to be unstable; old colonies sometimes sector and grow on YEPD plates. Great efforts have been made to address the dependency of the expression of \( chH1 \) in YRL4. This includes plasmid shuffling using a second plasmid containing either wild-type or disrupted \( chH1 \) gene. Nevertheless, due to the instability of this mutation, we were unable to conclusively prove that it was the \( chH1 \) gene alone that was responsible for suppression of this mutation. We detected a fivefold increased level of \( chH1 \) message in YRL4 compared to the level of \( chH1 \) message in wild-type controls (data not shown). This may indicate that the mutant required higher levels of H1 expression than originally expressed from this plasmid, which might be an important factor complicating the dependency analysis.

**Isolation and characterization of the \( P471 \) gene**

The gene complementing the glucose lethality of YRL4 was isolated from genomic libraries as described in Experimental procedures. One positive clone was obtained from the centromeric-based genomic library and nine were obtained from a YEP13-based genomic library. All clones also complement both the Sect\(^-\) and 5-FOA sensitive phenotypes of the mutation. Restriction mapping and Southern blotting analyses indicate that a 5 kb region is shared by all 10 clones (Fig. 1A). Further analyses revealed that the complementing activity resides within a 0.6 kb \( XhoI-XbaI \) fragment (Fig. 1A). The DNA sequence of a 1.5 kb \( SacI \) fragment (Fig. 1B) was determined and it overlaps with a sequence reported from a lambda clone from chromosome VI (accession no. Z46255). The longest open reading frame (ORF) in the 1.5 kb \( SacI \) fragment is 480 bp, starting 47 bp downstream of the \( XhoI \) site and ending 62 bp upstream of the \( XhoI \) site. When Northern blot analysis was performed using the 0.6 kb \( XhoI-XbaI \) fragment as a probe, a single band of about 700 nucleotides was detected in both wild-type and YRL4 RNA (data not shown). The predicted amino
acid sequence from this ORF shows no significant homology with histone H1. However, a domain of this protein (I97 to G152) shares significant homology with many previously identified acetyltransferases (Fig. 1C) and this gene was named PAT1 (for Putative Acetyl Transferase). The original pat1-1 allele and the parental PAT1 gene were isolated by gap repair, sequenced, and pat1-1 was found to have a D99N mutation in the homology domain indicating the relevance of this domain to Pat1p function.

To help establish the position of the translational initiation codon and the intron/exon structure of the PAT1 gene, we isolated cDNAs. To accomplish this, a yeast cDNA library was prepared in YES-R (Elledge et al. 1991). The RNA source of cDNA came from the strain Y133 (MATa, ura3-52, lys2-801, ade2-101, his3-∆200, Trp1∆). We obtained 1·108 independent lambda clones. The 0.6 kb XhoI-XbaI fragment was used to screen this library and 10 independent cDNA clones were obtained that coincide with this ORF. Two

![Figure 2](image-url)
of these were completely sequenced. The longer cDNA contained sequences from −13 to +548 according to the genomic sequences in Fig. 1B where +1 is the first ATG. The shorter cDNA contained sequences from +9 to +551. The PAT1 gene contains no introns and most likely utilizes the first ATG in the ORF as the initiation codon. This would generate a protein of 159 amino acids with predicted molecular weight of 19,203 Da.

To address the significance of this acetyltransferase domain and possible phylogenetic conservation of this gene we sought to isolate the S. pombe homologue by

**Figure 3** Aberrant morphology of Pat1p depleted cells. Abnormal cellular (phase contrast, left-hand column) and nuclear (DAPI stain, right-hand column) morphologies becomes apparent in YRL18 (pat1-Δ2::HIS3/pRL46) when shifted from YPG (a,b) to YPD (c–j) media for 24 h. Small arrows indicate aberrant nuclear segregation. Large arrows point to anucleated, large-budded cells. Bar = 20 μm.
complementation. An S. pombe cDNA library (Becker et al. 1991) was used to complement a pat1 null mutant (see Experimental procedures). Of 180 cDNA clones capable of suppressing pat1, all contained the same gene. The spPAT1 conceptual translation product is 356 amino acids (Fig. 2A) and the last 80 amino acids share 50% identity with the C-terminal half of the Pat1 protein (Fig. 2B). The homology extends beyond the putative acetyltransferase homology domain. Interestingly, the N-terminal third of the spPat1p is homologous to another S. cerevisiae gene YGL021 (Fig. 2B), a putative membrane protein on chromosome VII whose disruption shows no phenotype (Chen et al. 1991). No apparent membrane spanning domain is predicted from the PAT1 protein sequence.

Taken together, the sequence analysis suggests that PAT1 is not the yeast homologue of histone H1. Instead, the homology with other acetyltransferases, the conservation in the putative acetyltransferase domain with its S. pombe functional homologue, and the nonsense mutation detected in the original pat1-1 mutation all suggest that Pat1p is likely to be an acetyltransferase.

**PAT1 is essential for mitotic growth**

A pat1 disruption allele (pat1-Δ2::HIS3) was generated by one-step gene replacement in a diploid, YRL9, and used to examine whether PAT1 is required for mitotic growth. While the wild-type parental diploid gave rise to tetrads with four viable spores, YRL9 gave tetrads with only two viable spores which were always His+. The inviable spores germinated and each gave rise to an average of eight to 16 progeny cells. These data indicate that PAT1 is essential for mitotic growth. When PAT1, but not a disrupted pat1, on a LEU2 centromeric plasmid was introduced into YRL9, the resulting strain gave rise to tetrads that often contained greater than two viable spores. Every His+ spore was also Leu+, indicating that PAT1 is required for the viability of spores containing the pat1-Δ2::HIS3 allele.

The inviability of a pat1 deletion strain can also be rescued by the PAT1 cDNA under control of the GAL1 promoter. This was examined with both the longer (−13 to +548, pRL45) and shorter (+9 to +551, pRL46) cDNAs described above. The longer cDNA produces a protein of 159 amino acids, whereas the shorter cDNA produces a 148 amino acid protein lacking the first 11 amino acids. While both cDNAs rescue the spore inviability associated with the pat1 disruption allele on galactose media, they behave differently on glucose media. When streaked on YPD plates, the pat1 deletion strain that contains pRL45 (YRL17) could grow, while cells containing pRL46 (YRL18) could not.

**Depletion of Pat1p results in multiple phenotypes**

To address the function of PAT1, we examined the defects in YRL18 cells when Pat1p is gradually depleted by turning off the expression of the truncated form of PAT1 cDNA in the presence of glucose. Yeast cells were grown in YPG medium to the early logarithmic phase (OD₆₀₀ = 0.1), washed once with water, and diluted 20-fold in YPD medium. At different intervals after transfer to YPD medium, cells were fixed and stained with the DNA specific dye DAPI. The depletion of Pat1p led to multiple phenotypes which became apparent 20 h after cells were transferred to YPD media (Fig. 3). Cell sizes varied dramatically, with a high percentage (30%) of giant cells whose volumes enlarged by ≈100–200-fold (Fig. 3c–f). The majority of cells were still attached to each other, generating long chains and multiply budded cells. DAPI staining detected defects in nuclear segregation which gave rise to cells with either multiple nuclei (Fig. 3d,f,i) or lacking nuclei (Fig. 3j). These enlarged cells and multi-nucleated phenotypes have been observed in mutations which affect bud emergence (Bender & Pringle 1991). It was surprising to note that cytokinesis seemed to be proceeding in anucleated cells; anucleated cells with large buds were observed (large arrows in Fig. 3h,j), although we cannot rule out the possibility that these cells once had DNA, which was degraded prior to our analysis. In addition, it appears that cytokinesis occurred prior to the completion of nuclear migration and segregation in some cells, causing uneven nuclear division (see small arrows in Fig. 3e,f,g,i). However, in many cases observed here, cells seemed unable to complete cytokinesis when the nuclei were caught in the neck portion of two cells. Therefore, many cells were still connected, forming long chains. Whether this is an arrested point or a part of a dynamic process is still unclear. But the results obtained from a temperature sensitive mutation described later strongly suggest that this is an arrest point. The original pat1-1 mutant also exhibits the phenotype described here when it is transferred to glucose media, but less severely in galactose media. These results suggest that Pat1p may play a role in mitosis, concerning chromosome segregation and/or nuclear division. The phenotypic heterogeneity in Pat1p-depleted cells indicates that Pat1p is either involved in multiple processes or a process that is essential throughout the cell cycle.
Isolation of a temperature sensitive allele of the \textit{PAT1} gene

Phenotypes resulting from depletion experiments, although informative, lack the resolution that can be obtained by rapidly inactivating the activity of a protein using conditional mutants. Therefore, efforts were made to isolate \(T_s^-\) \textit{PAT1} alleles by \textit{in vitro} mutagenizing a plasmid containing a wild-type \textit{PAT1} gene followed by the plasmid shuffle method, as described in Rose & Fink (1987). One \(T_s^-\) allele (\textit{pat1-3}) was isolated from 20,000 clones screened. The plasmid pRL71, which contains \textit{pat1-3}, can confer the \(T_s^-\) phenotype on cells whenever it is reintroduced into a \textit{pat1-\(\Delta 2::HIS3\)} mutant strain. The \textit{pat1-3} mutant strain (YRL34) had slightly longer generation times (132 min) than wild-type (CRY1, 90 min) or phenotypically wild-type (YRL37, 111 min) control strains at 23°C, suggesting that \textit{pat1-3} mutants are partially defective, even at permissive temperatures.

The growth of the \textit{pat1-3} strain upon temperature shift to 37°C was examined and found to arrest growth prior to one complete cell doubling (data not shown). The survival of the wild-type and mutant cells at 37°C as a function of time was also examined. Wild-type cells tolerated the non-permissive temperature as expected, but cells expressing the \textit{pat1-3} allele lost viability at 37°C with a half-life of \(\approx 3\) h (data not shown).

The terminal phenotypes of the \textit{pat1-3} mutants at 37°C for 3.5 h were also examined (Fig. 4) and found to show great similarity to those obtained in the depletion experiment, but also revealed several unique phenotypes. For instance, the abnormality of chromatin structure and nuclear segregation were apparent in both cases, whereas giant cells and anucleated cells with large buds were not observed in the \textit{pat1-3} mutant. At 37°C, \textit{pat1-3} cells were not arrested at a defined cell cycle stage, although an increase of large-budded cells (G2-M phase, 42%) and a decrease of small-budded cells (S phase, 17%) were observed compared to wild-type controls (23% and 25%, respectively) grown at 37°C. Many cells were still attached to each other, forming groups of three, four, or even more cells, most of which had abnormal nuclei that were either fragmented or had a threaded appearance (large arrows in Fig. 4c,d). Nuclear segregation also appears to be affected and a high percentage of nuclei were either caught in the junction of two cells or improperly segregated, resulting in multiple nucleated (open arrowheads in Fig. 4e,g) or anucleated cells (closed arrowheads in Fig. 4f,h). It was evident that cytokinesis occurred prior to the completion of nuclear migration and segregation, resulting in cut-like phenotypes (small arrows in Fig. 4c,d,e,g; Hirano \textit{et al.} 1986; Samejima \textit{et al.} 1993). However, cytokinesis of the \textit{pat1-3} mutant was often incomplete, with the mother and daughter cells connected by a bridge of nuclear material.

The \textit{pat1-3} mutant is sensitive to benomyl

Since the above data indicate that Pat1p might be involved in nuclear segregation, a function in which tubulin is involved, \textit{pat1} mutants were tested for its sensitivity to an anti-microtubule drug, benomyl. It has been shown that microtubule-mediated processes, such as nuclear division, nuclear migration and nuclear fusion, are inhibited in the presence of benomyl (Quinlan \textit{et al.} 1980; Delgado & Conde 1984; Jacobs \textit{et al.} 1988) and that many tubulin or tubulin-related
mutants are supersensitive to benomyl in yeast (Umesono et al. 1983; Huffaker et al. 1988; Matsuzaki et al. 1988; Schatz et al. 1988; Stearns et al. 1990). The level of benomyl sensitivity is temperature dependent; yeast cells are more sensitive to benomyl at lower temperatures (Stearns et al. 1990).

If *PAT1* is involved in a microtubule-mediated process, the sensitivity to benomyl in *pat1* mutant cells might be exacerbated at conditions that are suboptimal for growth. In fact, the original *pat1-1* mutant (YRL4) was unable to grow on YPG plates containing 20 μg/mL benomyl at 30°C. However, its parental strain YMW1-6/pMW29H1 can grow in the presence of up to 30 μg/mL benomyl at 30°C. Introducing a wild-type *PAT1* gene to YRL4 restored its benomyl resistance to the wild-type level (data not shown), indicating that the sensitivity to benomyl in YRL4 is associated with the *pat1-1* mutation. We also examined the benomyl sensitivity of the *Ts−* *pat1-3* strain (YRL34) at a semi-permissive temperature (31.5°C, Fig. 5). At the permissive temperature (23°C), both YRL34 and the *Pat1* control YRL37 show similar sensitivity to benomyl; neither strain grows in the presence of 16 μg/mL benomyl. At 31.5°C, YRL34 grows very poorly at 16 μg/mL and dies at 20 μg/mL of benomyl, whereas YRL37 grows well at 16 μg/mL and poorly at 20 μg/mL of benomyl. This result suggests that under conditions where *pat1* mutants are stressed but still viable, they show increased benomyl sensitivity compared to wild-type cells. The sensitivity to benomyl for *pat1* is comparable to that for mutants which are defective in processes involved in nuclear division (Hoyt et al. 1991; Li & Murray 1991; Ursic & Culbertson 1991) but is lower than that for tubulin mutants (Matsuzaki et al. 1988; Schatz et al. 1988). This further suggests that Pat1p functions in a process where tubulin or related proteins are also involved.

**pat1** mutants have defects in spindle organization

Immunofluorescence was carried out with anti-tubulin antibodies to visualize microtubules in the *pat1-3* mutant. Figure 6 shows microtubule staining of the *pat1-3* strain at either permissive (23°C) or non-permissive (37°C) temperatures. At 23°C, the distribution of microtubules in *pat1-3* (Fig. 6a–c) is indistinguishable from that in the wild-type, in which ≈30% cells have elongated spindles (Pringle & Hartwell 1981; Kilmartin & Adams 1984). After a shift to 37°C for 4 h, YRL34 (*pat1-3*) has very few (<3%) cells with elongated spindles; the majority of cells have short spindles (small arrows in Fig. 6d–i) which were similar to those observed in cells treated with hydroxyurea, a DNA synthesis inhibitor (Byers & Goetsch 1975; Hartwell 1976). However, unlike spindles seen in hydroxyurea-treated cells that are usually located between mother cells and their large buds, spindles in YRL34 are seen even in unbudded and small budded cells (Fig. 6d–f). Further examination of those short spindles revealed that some were in fact two closely located spindle pole bodies (SPB, see closed arrowheads in Fig. 6g–i,j,l,n). The intranuclear spindles...
typically seen were either missing or greatly reduced here. Particularly revealing is the example shown in Fig. 6, panels j, l and n, in which nuclei have already separated and two SPBs are separated, yet are still at the proximal ends of the two nuclei. This is distinct from the wild-type cells at the same stage in which a long spindle connected by SPBs at distal ends of two nuclei is usually apparent (see large arrows in a–c). Panels k, m, and o show an example of a monopolar spindle in a dividing nucleus. Bar = 20 μm.

**Figure 6** Aberrant microtubular morphology in *pat1-3* mutant cells. YRL34 (*pat1-3*) *pat1-1Δ2::HIS3*) incubated at either 23°C (a,b,c) or 37°C (d–o) were prepared for immunofluorescence using anti-tubulin antibodies (YOL1/34) and FITC-conjugated second antibodies. Cells were examined by either phase contrast (a,d,g,j,k), FITC-fluorescence (c,f,i,n,o) or DAPI (b,e,h,l,m) fluorescence microscopy. The small arrows point to the short spindles observed in the *pat1-1-3* mutation (d–i) at the nonpermissive temperature. This is different from the elongated spindles seen in wild-type or *pat1-1-3* of the same stage at permissive temperatures (large arrows in a–c). The closed arrowheads indicate cells with two separated SPBs (g–i,j,l,n). The open arrowhead points to a mis-orientated spindle.
pat1-3 is abnormal in nuclear segregation and is more sensitive to benomyl.

A requirement for Pat1p function in G1 and M phases

The pat1-3 Ts– mutants do not arrest at a defined point of the cell cycle when shifted to nonpermissive temperatures. However, there is an increase in the large-budded cells and a decrease in the small-budded cells, suggesting that P4T1 might be required for the S phase in addition to mitosis. We performed FACS analyses to examine the DNA contents of the pat1-3 mutant arrested at a nonpermissive temperature. When an asynchronous pat1-3 culture is shifted to 37°C for 3.5 h, the majority of cells have DNA contents either greater than 2C or less than 1C, suggesting the occurrence of aberrant chromosome segregation (data not shown). This was consistent with the observation of ‘cut’ cells. To more precisely determine the defects at particular cell cycle stages, this analysis was also performed with cells synchronized with α-factor at 23°C. After release from α-factor arrest, cells were either transferred immediately or allowed to recover at 23°C for different intervals before transfer to 37°C and incubation for 3.5 h. Control strains proceeded through the cell cycle synchronously after they were released from the α-factor arrest but gradually lost the synchrony after the second cycle. Figure 7 shows the FACS profiles of a wild-type control arrested with α-factor (a) and released from α-factor to the 37°C for 3.5 h (b). It was clear that cells are arrested with 1C DNA content in the presence of α-factor but lost the synchrony (the majority of cells are still at the G2 of the third cycle) after release from α-factor and incubated for 3.5 h at 37°C. When pat1-3 cells were transferred to 37°C immediately after release from the α-factor arrest, their DNA remained unreplicated (Fig. 7c,d) and cells were still schmooed after 3.5 h (data not shown) suggesting that P4T1 is required for exiting the α-factor block, passage through START, or the initiation of DNA synthesis. When cells were allowed to recover at 23°C for 75 min (they appeared to be small budded and have initiated DNA synthesis at this point), they became arrested as large-budded cells, the majority of which have a G2 DNA content after 3.5 h at 37°C (Fig. 7n), suggesting that P4T1 is also required for mitosis. If the recovery time at 23°C was shorter than 75 min (Fig. 7e,g,i,k), the majority of cells showed DNA contents intermediate between 1C and 2C after 3.5 h at 37°C (Fig. 7f,h,j,l). This result suggests that P4T1 is also required for the progression of DNA synthesis. The FACS analysis of pat1 Ts– mutants released from α-factor arrest point is consistent with other analyses suggesting that P4T1 is required for multiple points of the cell cycle: to exit the α-factor block, progression of DNA synthesis, and mitosis.
Isolation of cold sensitive suppressors for the 
\textit{pat1-3} Ts\textsuperscript{−} mutation

We have isolated three spontaneous cold sensitive (Cs\textsuperscript{−}) suppressors to the Ts\textsuperscript{−} \textit{pat1-3} mutation (see Experimental procedures). One is a dominant suppressor that is recessive for the Cs\textsuperscript{−} phenotype. The other two (YRL40 and YRL41) belong to the same complementation group and are recessive for both Cs\textsuperscript{−} and suppression phenotypes. We isolate genomic clones that complement the Cs\textsuperscript{−} phenotype of YRL40 and show that the gene responsible for the complementation is a previously identified gene \textit{SAC1} (suppressor of actin, Novick et al. 1989). \textit{SAC1} encodes an integral membrane protein that localizes to the yeast Golgi complex and ER (Whitters et al. 1993). It was shown by gene disruption that the function of \textit{SAC1} is only essential at temperatures below 17°C (Novick et al. 1989). Although \textit{sac1} was originally isolated as an allele-specific Cs\textsuperscript{−} suppressor to \textit{act1-1} Ts\textsuperscript{−} mutation it was also later shown to be capable of suppressing three mutations that are in the secretory pathway, \textit{sec14}, \textit{sec6} and \textit{sec9} (Cleves et al. 1989). We have generated a \textit{sac1} deletion allele and shown that it is cold sensitive as described by Novick et al. (1989), and that it fails to suppress the \textit{pat1-3} temperature sensitivity at 37°C (Experimental procedures). \textit{sac1} null mutations also fail to suppress \textit{act1-1} mutants. The isolation of \textit{sac1} as a suppressor of \textit{pat1} potentially links Pat1p to actin and to secretory-mediated processes.

Discussion

In this study, we have isolated a yeast mutation in the gene \textit{PAT1} using a colony-colour-sectoring assay for mutations whose growth depends on the expression of a vertebrate histone H1. \textit{pat1-1} can be partially suppressed by the presence of a plasmid expressing H1, although we cannot explain how this suppression occurs. We have shown that \textit{PAT1} is essential for mitotic growth and that it encodes a small novel protein which shares homology with several acetyltransferases. Mutant cells depleted of Pat1p show aberrant cellular and nuclear morphology. We have also shown using a temperature sensitive mutation (\textit{pat1-3}) that \textit{PAT1} functions at multiple steps in the cell cycle.

\textit{PAT1} is required at multiple steps in the cell cycle

Our analysis of \textit{pat1} mutants suggests that \textit{PAT1} is required at multiple points in the cell cycle: to exit the α-factor block, the progression of DNA synthesis, and proper mitosis. \textit{pat1-3} mutant cells do not undergo DNA replication and remain shmooed if transferred immediately to 37°C after release from α-factor. DNA replication can occur and be completed, however, if the \textit{pat1-3} mutant cells are allowed to recover at permissive temperatures for 75 min before being shifted to 37°C. At 75 min, DNA synthesis is already initiated. Nevertheless, the initiation of DNA synthesis is not sufficient, because \textit{pat1-3} mutant cells recovered at permissive temperatures for 60 min show a similar extent of DNA synthesis but fail to complete DNA synthesis when transferred to 37°C. Both microscopic examination using DAPI and FACS analyses suggest that Pat1p is also required at mitosis for chromosome segregation and nuclear division. The pleiotrophic phenotype of the \textit{pat1} mutant is superficially similar to the phenotypes caused by defects in protein synthesis (Burke & Church 1991). Treatment with cycloheximide suggests that protein synthesis is required at multiple steps in the cell cycle, including completion of G1, initiation of DNA synthesis, progression from the end of DNA synthesis to nuclear division, completion of cytokinesis and cell separation. However, the nuclear catastrophe observed during mitosis and chromosome segregation defects indicated by FACS analyses in the \textit{pat1-3} mutant are not observed in cycloheximide treated yeast cells. Therefore, although \textit{PAT1} is involved in multiple steps in the cell cycle, it is unlikely that \textit{PAT1} functions by affecting protein synthesis.

Several mutants in \textit{S. pombe} show a ‘cut’ phenotype like that observed in \textit{pat1-3} Ts\textsuperscript{−} mutants. These include those defective in sister chromatid separation such as topoisomerase II mutants (Goto & Wang 1985; Uemura & Yanagida 1986) and those that produce premature or inappropriate mitosis such as severe wee mutants (Enoch & Nurse 1990) and certain checkpoint defective mutants when DNA replication is delayed (Enoch et al. 1991; Al-khodairy & Carr 1992; Rowley et al. 1992). Presumably, \textit{S. cerevisiae} topoisomerases activate a checkpoint that normally prevents cut phenotypes. \textit{pat1} mutants on the other hand are defective in some aspect of coordination of chromosome separation and nuclear division with cytokinesis. While some checkpoints may be defective, the S phase checkpoint appears to be intact because \textit{pat1} mutants are not sensitive to hydroxyurea which blocks DNA replication. It is curious that in a subset of cells observed using a Pat1p depletion protocol there appears to be reduced cytokinesis, with groups of cells connected to each other while others in the Ts\textsuperscript{−} \textit{pat1} mutants show this
Cut phenotype. Clearly this points to a very complicated set of defects in pat1 mutants.

The overall phenotypes of the pat1 mutation resemble the phenotype of the double mutants in type I (top1) and type II (topII) topoisomerases in yeast. The complete defect in controlling the superhelical density can only be observed in the top1-topII double mutants whose phenotype is strikingly different from those of either the single mutant (Uemura & Yanagida 1984; Goto & Wang 1985). At nonpermissive temperatures, the Ts- top1-topII double mutant cells have diminished DNA, RNA and protein synthesis and are quickly arrested at various stages of the cell cycle with defects in DNA synthesis, mitosis, nuclear division and chromosome segregation (Uemura & Yanagida 1984, 1986).

On the basis of these similarities between phenotypes of pat1 mutants and topi-topII double mutants, it is plausible to suggest that the PAT1 gene may somehow be involved in the same pathway as topoisomerase I and II to influence DNA superhelicity.

**Does PAT1 encode an acetyltransferase?**

PAT1 shows no resemblance to histone H1 in either amino acid composition or secondary structure. However, we can not rule out the possibility that PAT1 is indirectly performing a role similar to histone H1 in the regulation of higher order chromatin organization. In support of a chromatin role, cells in the Pat1p deletion experiments had chromatin with an unusual ‘threaded’ appearance, indicating a possible role for Pat1p in higher-order chromatin structure; such a role could explain the pleotrophic phenotypes associated with pat1 mutants through modulation of gene expression in a chromatin environment.

Several lines of evidence suggest that Pat1p is an acetyltransferase. First, it has significant homology with the conserved domain of several known acetyltransferases such as Hat1p (Kleff et al. 1995), Ard1p (Mullen et al. 1989; Lee et al. 1989), Mak3p (Tercero & Wickner 1992), and several bacterial acetyltransferases.

Secondly, the original pat1-1 mutation has a D99N change within this conserved domain, suggesting that this domain is important for Pat1p function. Finally, an S. pombe gene that was isolated by its ability to complement the *S. cerevisiae* pat1 deletion mutant also contains a high conservation of the putative acetyltransferase domain. However, the spPat1p might have additional functions or might function in a different cellular compartment, since its N-terminal third is homologous to another *S. cerevisiae* protein, a putative membrane protein (Chen et al. 1991). Efforts have been made to detect the acetyltransferase activity with HA-tagged Pat1p. While the anti-HA antibody was able to immunoprecipitate HA-Pat1p, no activity was so far detected using either histones or tubulin as substrates (data not shown). However, this analysis suffers from an absence of knowledge of the proper substrate for this protein and whether the antibody recognizes or inhibits the active form of Pat1p.

Acetylation has been implicated in a wide variety of biological processes. Acetylation of the N-terminal NH2 occurs in almost all proteins co-translationally and this modification appears to be essential for viability in *S. cerevisiae* (Kulkarni & Sherman 1994). Three N-terminal acetyltransferases have been identified in *S. cerevisiae*, ARD1/NAT1, NAT2 and MAK3 (Lee et al. 1989; Mullen et al. 1989; Park & Szostak 1992; Tercero & Wickner 1992; Kulkarni & Sherman 1994). Mutations in nat2 cause lethality whereas mutations in either nat1 or ard1 activate the silent mating type locus (HML) and prevent cells from entering into G0 (Whiteway & Szostak 1985; Lee et al. 1989; Mullen et al. 1989; Kulkarni & Sherman 1994). In addition, N-terminal acetylation has been shown to be important for the processing of actin protein and the polymerization of striated alpha tropomyosin as well as its ability to bind to F-actin (Hitchcock-DeGregori & Heald 1987; Cook et al. 1991; Urbanickova & Hitchcock-DeGregori 1994).

A second kind of acetylation occurs post-translationally on the NH2 group of internal lysines. One of the better studied examples of internal acetylation occurs on the N-terminal domains of all core histones. Strong correlations have been made between histone acetylation and histone deposition (Allis et al. 1985; Csordas 1990), transcriptional activity of certain genes (Hebbes et al. 1988; Csordas 1990; Wolffe 1994; Brownell et al. 1996; Wolffe & Pruss 1996b), and histone displacement during mammalian spermatogenesis (Christensen & Dixon 1982). The lysines (K5, K8, K12, K16) that can be subjected to acetylation on the N-terminal domain of histone H4 are highly conserved in evolution. Cells with substitution of asparagine for all lysines in this domain are not viable (Megee et al. 1990), whereas substitution of arginine for these lysines results in either very slow growing (Durrin et al. 1991) or lethal (Megee et al. 1990) phenotypes. Substituting glutamine for all four lysines is not lethal, but confers several phenotypes, including prolonged S and G2+M periods of the division cycle, mating sterility and temperature sensitive growth (Megee et al. 1990). Recently, it was shown that certain patterns of acetylation are essential for the
efficient silencing of transcription at the mating type loci: K16 must be deacetylated and at least K5 or K12 must be acetylated (Park & Szostak 1990; Braunstein et al. 1996). In addition, heterochromatin in yeast and flies is enriched in histone H4 that is acetylated at a specific lysine residue, K12 (Braunstein et al. 1996; Turner et al. 1992). Taken together, these results suggest that while the positive charge contained in the N-terminal domains of H4 (Johnson et al. 1990; Megee et al. 1990; Park & Szostak 1990) and H3 (Thompson et al. 1994) contributes to the formation and/or stability of heterochromatin in yeast, acetylation of specific lysine residues (K12) also plays an important role. Presumably acetylation influences heterochromatin formation through protein–protein interactions that are only beginning to be defined (Hecht et al. 1995).

Although we have not shown that Pat1p exhibits acetyltransferase activity, it is possible that Pat1p is a histone acetyltransferase in S. cerevisiae. To date, none of the current histone acetyltransferases that have been cloned are essential. Given the increasingly clear involvement of histone acetylation/deacetylation in the activation/repression of at least some yeast genes (Brownell et al. 1996; Taunton et al. 1996), it is not difficult to imagine that mutations in histone acetyltransferases can indirectly affect multiple cellular functions through changes in gene expression. We do not know why the expression of a vertebrate histone H1 suppresses the original pat1-1 (but not pat1-Δ2::HIS3 nor pat1-3) mutation, although a link to chromatin is suggested. Both histone H1 and acetylation on the core histone amino-terminal domains are thought to modulate the higher order chromatin organization, and several studies have shown that H1-mediated reactions are affected by the core histone amino-terminal domains and their acetylation (Ridsdale et al. 1990; Perry & Annunziato 1991; Juan et al. 1994). An intriguing possibility is that Pat1p functions in the acetylation of lysine 12, which appears to be associated with heterochromatin in yeast (Braunstein et al. 1996). Inability to acetylate lysine 12 and establish proper heterochromatin formation might result in the 'threaded' and 'diffused' chromatin observed in the pat1 mutants. In this scenario, expression of a vertebrate H1 might help to counteract the compromised organization of heterochromatin caused by the lack of acetylation at lysine 12 in H4.

**Suppression of the pat1-3 by sac1 mutations**

Our suppressor analysis on the pat1-3 Ts+ mutation has identified two sac1 Cs+ mutations. sac1 was initially identified as an allele-specific suppressor to the act1-1 Ts+ mutation, and is distinguishable from other sac mutations in its capability to suppress some secretion mutants in addition to act1-1 (Cleves et al. 1989; Novick et al. 1989). It is an integral membrane protein in Golgi and ER (Whitters et al. 1993). We do not at present understand why mutations in SAC1 suppress the pat1-3 mutation, but would like to speculate on two possibilities. It was proposed that SAC1 might play a role in biological events whereby secretory and cytoskeletal activities are coordinated. Although we have not detected abnormalities in the actin network in the pat1-3 mutants, it does affect the microtubule organization and benomyl sensitivity. It has been shown in many cases that the actin and microtubule machinery interact (Schmit & Lambert 1988; Lillie & Brown 1992; Pedrotti et al. 1994; Ursic et al. 1994), therefore it is possible that sac1 also suppress some microtubule-related defects. It has been suggested that acetylation increases the stability of microtubule and rabbit skeletal troponin C and also increases the interaction of the latter with other troponin components (Maruta et al. 1986; Grabarek et al. 1995). Perhaps pat1 mutants are affecting the ability of these two networks to properly interact. Intriguingly, Smy1 is a microtubule-based motor protein which was isolated as a multi-copy suppressor of a mutation in an actin-based motor protein, Myo2, whose function has been implicated in the process of polarized secretion in yeast (Lillie & Brown 1992).

A second possibility is that although Pat1p does not contain an obvious transmembrane domain, it might associate with membrane through interaction with a membrane protein. The interaction might allow Pat1p to function in a particular cellular compartment or facilitate the assembly of Pat1p into a complex. Mutations in pat1-3 might affect the association with this hypothetical membrane protein and disable Pat1p from functioning in the proper cellular compartment. Since mutations in sac1 can suppress—and in some cases bypass—several mutations in the secretory pathway, they might suppress pat1-3 by allowing the interaction between Pat1p and the proper membrane protein or by allowing Pat1p to be located in the proper cellular compartment. This notion is supported by the interesting observation that spPat1p consists of two separate domains, each of which is homologous to a different S. cerevisiae protein. The N-terminal third is homologous to a putative transmembrane protein, YGL021, and the C-terminal third is to Pat1p. Nevertheless, YGL021 deletion does not result in any detectable phenotype (Chen et al. 1991).
### Table 1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMW1-6</td>
<td>MATα ade2-1 ade3Δ his3-11, 15 leu2-3, 112 snr3-1</td>
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</tr>
<tr>
<td>CRY1</td>
<td>MATa can1-100 ade2-1 his3-11, 15 leu2-3, 112 trp1-1 snr3-1</td>
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<tr>
<td>CRY1U6</td>
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<td>CRY1L</td>
<td>pUN105</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>YRL1</td>
<td>pUN105</td>
<td></td>
</tr>
<tr>
<td>YRL4</td>
<td>MATa YMW1-6 pat1-1 (URA3 ADE3)</td>
<td></td>
</tr>
<tr>
<td>YRL9</td>
<td>MATa/MATα CRY3 PAT1/pat1-Δ2::HIS3</td>
<td></td>
</tr>
<tr>
<td>YRL17</td>
<td>pRL45</td>
<td>As CRY1 but MATα pat1-Δ2::HIS3 (URA3 GAL1-PAT1†)</td>
</tr>
<tr>
<td>YRL18</td>
<td>pRL46</td>
<td>As CRY1 but MATα pat1-Δ2::HIS3 (URA3 GAL1-PAT1‡)</td>
</tr>
<tr>
<td>YRL26</td>
<td>pRL57</td>
<td>As CRY1 but MATα pat1-Δ2::HIS3 (URA3 PAT1)</td>
</tr>
<tr>
<td>YRL30</td>
<td>pRL71</td>
<td>As CRY1 but MATα pat1-Δ2::HIS3 (LEU2 pat1-3)</td>
</tr>
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<td>YRL34</td>
<td>pRL71</td>
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<td>YRL37</td>
<td>pRL58</td>
<td>As CRY1 but pat1-Δ2::HIS3 (LEU2 PAT1)</td>
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<td>pRL58</td>
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</tr>
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</tr>
<tr>
<td>YRL98</td>
<td>pUN105</td>
<td></td>
</tr>
<tr>
<td>YRL101</td>
<td>pRL58</td>
<td>As CRY1 but pat1-Δ2::HIS3 (LEU2 PAT1)</td>
</tr>
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<td>pRL58</td>
<td>As CRY1 but pat1-Δ2::HIS3 (LEU2 pat1-3)</td>
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<td>YRL127</td>
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</tr>
</tbody>
</table>

* Zieler et al. 1995.  
† PAT1 cDNA.  
‡ Shorter version of PAT1 cDNA.  
Markers in parentheses reside on the plasmid.

Pat1p was dependent on interaction with a membrane protein, it would have to be a protein other than YGL021.

### Experimental procedures

**Strains, media and microbial techniques**

Strains used in this study are listed in Table 1. Yeast media were prepared as in Sherman et al. (1986). When appropriate, 2% galactose was used in place of 2% glucose. Unless otherwise stated, all yeast strains were grown at 30°C. Benomyl was dissolved in dimethylsulphoxide (DMSO) as a 30 mg/mL stock. Yeast transformations were performed by the lithium acetate method (Ito et al. 1983). JM107 was used as a host for all plasmid constructions and amplification. XL1Blue was used for the preparation of single stranded DNA, and LE392 was used as a host for the λ phage (Elledge et al. 1991).

To isolate the pat1-1 allele, YMW1-6 containing plasmid pMW29H1 was mutagenized with EMS to 60% survival, as described in Sherman et al. (1986), resuspended in YPG medium to a density of 2 x 10⁷ cells/mL, incubated for 4 h and plated on YPG plates at a density of 700 colonies/150 mm plate, and

allowed to grow at 30°C for 5–7 days before identifying homogeneously red colonies.

The pat1-Δ2::HIS3 allele was generated by transforming a pat1 disruption construct, pRL9 (see below) cleaved with SacI, into the diploid CRY3 and selecting for histidine prototrophy. Homologous recombinants were verified by Southern analysis. The resulting strain YRL9 is heterozygous for the disrupted pat1 locus (pat1-Δ2::HIS3/PAT1) and was sporulated and examined for the viability of its progeny by tetrad analyses.

The pat1-3 Ts– allele was isolated by the in vitro mutagenesis of pRL58 with hydroxylamine and detected using a plasmid shuffle protocol according to procedures described previously (Rose & Fink 1987; Sikorski & Boeke 1990). Mutagenized pRL58 was transformed into YRL26 (pat1-Δ2::HIS3) carrying PAT1 on a centromeric URA3 plasmid (pRL57) treated as described (Sikorski & Boeke 1990). Only one temperature sensitive clone, pat1-3, was obtained from 20 000 colonies. The plasmid (pRL71) carrying the pat1-3 mutation was recovered from yeast and provides temperature sensitivity when reintroduced into a PAT1 disrupted strain.

### Plasmid constructions and DNA manipulation

Plasmids related to this study are listed in Table 2. DNA
Table 2 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Yeast marker</th>
<th>Relevant features and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMW29H1</td>
<td>URA3</td>
<td>GAL1-chH1, ADE3, CEN4*</td>
</tr>
<tr>
<td>pUN105</td>
<td>LEU2</td>
<td>CEN4†</td>
</tr>
<tr>
<td>pRL45</td>
<td>URA3</td>
<td>GAL1-PAT1‡, CEN4</td>
</tr>
<tr>
<td>pRL46</td>
<td>URA3</td>
<td>GAL1-PAT1§, CEN4</td>
</tr>
<tr>
<td>pSS1.5</td>
<td>LEU2</td>
<td>1.5 kb Sall fragment containing PAT1, CEN4</td>
</tr>
<tr>
<td>pRL57</td>
<td>URA3</td>
<td>0.6 kb XhoI-XhoI fragment containing PAT1, CEN4</td>
</tr>
<tr>
<td>pRL58</td>
<td>LEU2</td>
<td>0.6 kb XhoI-XhoI fragment containing PAT1, CEN4</td>
</tr>
<tr>
<td>pRL55</td>
<td>URA3</td>
<td>GAL-chH1-lacZ, CEN4</td>
</tr>
<tr>
<td>pRL56</td>
<td>URA3</td>
<td>similar to pRL55, lacZ was fused out of frame with chH1, CEN4</td>
</tr>
<tr>
<td>pRL71</td>
<td>LEU2</td>
<td>pat1-3, CEN4</td>
</tr>
<tr>
<td>pRL9</td>
<td></td>
<td>pat1-Δ2::HIS3, CEN4</td>
</tr>
<tr>
<td>pRL131</td>
<td>URA3</td>
<td>2.4 kb genomic fragment containing SAC1, CEN4</td>
</tr>
<tr>
<td>pRL149</td>
<td>LEU2</td>
<td>an integration plasmid containing GAL1-PAT1</td>
</tr>
</tbody>
</table>

* Provided by Mark Walberg.
† Elledge & Davis 1988.
‡ Longer PAT1 cDNA.
§ Shorter PAT1 cDNA.

Manipulations are essentially as described in Maniatis et al. (1982). The chicken histone H1 gene 11 L (chH1) contains no intron and was amplified from chicken genomic DNA by polymerase chain reaction (PCR) with the following two oligonucleotides: TCTCCTCCAGCAGCTCCGACATGTC and GCCGGGTCCAGGGAAATTCTCCCACAAG (Coles et al. 1987). This 711 bp fragment was digested with XhoI and EcoRI, whose recognition sites are underlined in the 5' and 3' oligonucleotides, respectively, and subcloned into XhoI-EcoRI cleaved Bluescript pBSKSII+. The chH1 clone was sequenced to ensure that no errors were generated by PCR. The chH1 gene was then digested with XhoI, filled in with dNTPs and Klenow, digested with XhoI, and cloned into the Smal-XhoI digested pMW29 (kindly provided by Mark Walberg, VT Southwestern, Dallas, Zieler et al. 1995) to make pMW29H1.

The molecular characterization of genomic DNA containing the PAT1 locus was performed with a genomic clone isolated from the centromeric-based library. The 1.5 kb Sall fragment was subcloned into pUN105 (Elledge & Davis 1988) giving rise to pSS1.5. pRL58 was created by an internal deletion of the XhoI fragment from pSS1.5 which removes the sequence downstream of the XhoI site to a XhoI site in the vector. The SacII-XhoI fragment was subcloned from pRL58 into SacII and XhoI digested pUN95 (Elledge & Davis 1988), giving pRL57. The deletion construct, pRL9, was generated by replacing the Nol-BclI fragment on pSS1.5 with a 2.5 kb HIS3 cassette from pJAS0 containing the HIS3 and the Tn 5 neo genes (Allen & Elledge 1994).

PAT1 cDNA clones from the yeast cDNA library were subcloned as XhoI fragments into XhoI-cleaved pBSKSII+ for sequencing or XhoI-cleaved pSE936 to place them under GAL1 control.

Sequencing was performed by the generation of a series of nested deletion clones generated by DNasel as described in Maniatis et al. (1982). Single-stranded DNAs were prepared following the methods of Zagursky & Berman (1984), using the helper phage R408 (Russe1 el al. 1986). All sequencing was performed on both strands by the dideoxy chain termination method (Sanger et al. 1977).

The GAL1-PAT1 cDNA was purified as a SacI-XhoI fragment from pRL46 (lacking the first three amino acids of Pat1p) and subcloned onto the Smal and XhoI digested pClac28, an integration vector (Gietz & Sugino 1988). The resulting plasmid pRL149 was digested with EcoRV and transformed the yeast strain YRL26. The yeast transformant was streaked on SC-Leu galactose plates, followed by 5-FOA galactose plates to remove the plasmid pRL57. The resulting strain YRL127 contains a disrupted pat1 gene (pat1-Δ2::HIS3) and a GAL1-PAT1 cDNA integrated to the LEU2 locus.

Isolation of the PAT1 gene

YRL4 (pat1-1) was transformed with two LEU2 genomic libraries, one in p366, LEU2 CEN, or YEP13 LEU2 2µ (Nasmyth & Reed 1980) yeast shuttle vectors. Transformants were selected on SC-leucine glucose plates. Approximately 100 000 transformants were screened from each library. One PAT1 clone from the p366-based library and nine from the YEP13 library were isolated, all contained the same gene and were able to complement all the pat1 phenotypes.

To ensure the phenotype of YRL4 is indeed due to defects in the PAT1 gene we PCR amplified the PAT1 locus from YRL4 and showed that it fails to complement a pat1 deletion strain to the wild-type level. The PAT1 locus from either a wild-type
strain (YM1-6) or YRL4 was amplified using oligonucleotides flanking the \textit{PAT1} ORF. These two PCR products were separately digested with \textit{Sal}I and \textit{Xho}I and subcloned to \textit{Sac} \textit{I} digest \textit{pUN105}. Five independent clones were obtained from each PCR and transformed into YRL26 (\textit{pat1-\Delta2::HIS3}, \textit{pRL57}), \textit{pRL58} and \textit{pUN105} were served as positive and negative controls, respectively. Transformants were selected on SC-Leu-Ura plates and, from each clone, four transformants were streaked on 5-FOA plates to test whether the new plasmid can replace \textit{pRL57} and complement \textit{pat1} deletion. While all clones grow equally well on the SC-Leu-Ura plates, only clones from wild-type DNA and \textit{pRL58} can fully substitute \textit{pRL57} and grow well on 5-FOA plates. All clones from YRL4 DNA only partially substitute \textit{pRL57} and grow poorly on 5-FOA plates; both the number and size of colonies on 5-FOA were greatly reduced. This result indicates that YRL4 has defects in the \textit{PAT1} locus instead of having another mutation that is suppressible by the \textit{PAT1} gene.

\section*{Isolation of \textit{Schizosaccharomyces pombe} \textit{PAT1} homologue by functional complementation}

\textit{YRL127} contains \textit{pat1-\Delta2::HIS3} and survives due to the \textit{GAL1-PAT1} cDNA integrated in \textit{LEU2} locus. \textit{YRL127} is unable to grow on glucose plates but grows well on galactose plates. An \textit{S. pombe} cDNA library under \textit{ADH} promoter control in \textit{pDB20 (URA3, 2\mu, Becker et al. 1991)} was transformed in \textit{YRL127} and the transformant was selected on SC-Ura glucose plates. From over 200000 transformants screened, 180 positive clones were obtained that suppress the inviability of \textit{YRL127} on glucose in a 5-FOA-dependent manner. Hybridization analysis showed that all the 185 clones belonged to the same gene. Further restriction mapping was carried out on nine clones and showed that they all contain a cDNA insert of \( \approx 1.3 \text{ kb} \) and all have an \textit{HindIII} site that is 1 \text{ kb} from one end. Only one cDNA clone was sequenced. The sequence was confirmed by the \textit{S. pombe} sequencing project from the Sanger Center.

\section*{\textit{pat1-3} suppressor screen}

The spontaneous reversion rate for the \textit{pat1-3} Ts\textsuperscript{−} allele (YRL30) at 37°C is \( \approx 3.5 \times 10^{-7} \). Two hundred independent \textit{YRL130} colonies grown at 23°C were inoculated into 200 individual 3 mL liquid YPD media to a density of around 1 \( \times 10^7 \text{ /mL} \). From each individual culture, 1 \( \times 10^7 \) cells were spun down and plated on to a 10 mm YPD plate. After 2 days incubation at 37°C, colonies grown were replica-plated on to two YPD plates, and grown at 23 and 37°C, respectively. Colonies that grow at 37°C, but not 23°C contain candidate Cs\textsuperscript{−} suppressors of the \textit{pat1-3} allele. Only one clone from each original plate was analysed further. Three clones that belong to two complementation groups were isolated. The first group is a dominant suppressor but is recessive for the Cs\textsuperscript{−} phenotype and was not further characterized. The second group contains two members and are named YRL40 and YRL41.

\section*{Characterization of the \textit{SAC1} clones}

Genomic clones complementing the Cs\textsuperscript{−} lethality of YRL40 were isolated from a yeast centromeric genomic library. A 2.4 kb \textit{Xho}I fragment was cloned to \textit{Xho}I-cleaved \textit{pUN70 (Elledge \\& Davis 1988)} resulting in \textit{pRL131} which was shown to be sufficient for the complementation. Partial sequencing analysis revealed that this genomic clone contains a previously identified gene, \textit{SAC1} (Whitten et al. 1993). A frame shift introduced to the \textit{SAC1} ORF on \textit{pRL131} eliminated its ability to complement \textit{YRL40}. The \textit{sacl} disruption allele was generated by replacing the 1.3 kb \textit{Xho}I-BamHI fragment with a 2.4 kb \textit{BamHI-XhoI URA3 cassette from pJA53} (Allen \\& Elledge 1994). A \textit{sacl} deletion mutant (YRL130) is cold sensitive at temperatures below 17°C, consistent with results described by Novick et al. (1989). To analyse whether a \textit{sacl} deletion mutation can suppress the temperature sensitivity of \textit{pat1-3}, YRL130 was mated with \textit{YRL30 (pat1-\Delta2::HIS3/pat1-3)} and diploids were selected on SC-Leu-Ura plates. These diploids were sporulated at 23°C and the viable spores that are Ura\textsuperscript{−}Leu\textsuperscript{−}His\textsuperscript{−} are tested for their viability at 37°C. Of 10 such spores analysed, none were able to grow at 37°C, suggesting that a \textit{sacl} deletion mutation cannot suppress the \textit{pat1-3} temperature sensitivity. Tetrads analyses were also performed to ensure that the Cs\textsuperscript{−} phenotype in YRL40 is linked to the \textit{SAC1} locus. YRL40 was mated to CRY1U6 and the diploid was sporulated. Cs\textsuperscript{−} spores that were His\textsuperscript{−}Leu\textsuperscript{−}Ura\textsuperscript{−} were mated to CRY1L to generate YRL98 (Cs\textsuperscript{+}/Cs\textsuperscript{−}). YRL98 was then transformed with a \textit{sacl} disruption allele which was generated by replacing the 1.3 kb \textit{XhoI-BamHI} fragment with a 2.5 kb \textit{BamHI-SalI HIS3 cassette from pJA50} (Allen \\& Elledge 1994). His\textsuperscript{−} transformants were selected and sporulated. Two His\textsuperscript{−} transformants, YRL101 and YRL102, were sporulated for tetrad analysis. Since it is not known whether the \textit{sacl} disruption occurs on the wild-type or \textit{sacl-2} allele, there were two possible outcomes of sporulation. If the original Cs\textsuperscript{−} mutant is in the \textit{SAC1} gene, we would obtain either 4:0 or 2:2 Cs\textsuperscript{−}/Cs\textsuperscript{−} outcomes of sporulation. If the original Cs\textsuperscript{−} mutant is in the \textit{SAC1} gene, we would obtain either 4:0 or 2:2 Cs\textsuperscript{−}/Cs\textsuperscript{−} segregation in each tetrad dissected. If the Cs\textsuperscript{−} phenotype is due to a mutation unlinked to \textit{SAC1}, then we would observe both DT, NPT and TT. Twenty tetrads for each strain were analysed from YRL101 and YRL102 and they all have only two Cs\textsuperscript{−} spores, which are also always His\textsuperscript{−}, indicating that the Cs\textsuperscript{−} phenotype in YRL40 is linked to the \textit{SAC1} locus.

\section*{Immunofluorescence and microscopy}

The immunofluorescence procedure described by Kilmartin \\& Adams (1984) was followed with the exception that cells were fixed in formaldehyde for 2 h at 30°C and cell walls were subsequently removed by treatment with 50 \( \mu \text{g/mL Zymolyase 100T} \) for 1 h at 37°C. Rat monoclonal anti-tubulin antibody YOL 1/34 and FITC-conjugated goat anti-rat IgG antiserum were obtained from Accurate Chemical \\& Scientific Corporation (Westbury, NY). After incubation with the secondary antibody, cells were stained with 1 \( \mu \text{g/mL DAPI} \) for 5 min. In cases where only DAPI staining was required, cells were fixed in 95% ethanol, incubated 1 \( \mu \text{g/mL DAPI} \) for 5 min at room temperature. Cells were then washed five times with water, resuspended in 100%...
methanol, and applied to poly-lysine coated slides. Preparations were viewed on a Zeiss Axioscope equipped for epifluorescence microscopy (Carl Zeiss, Germany).

Cell-cycle synchronization and flow cytometry

Cells were grown in YPD at pH 3.9 to an OD_{600} of 0.2 at 23°C. α-factor was added to a final concentration of 10 μM/mL. Cells were incubated for 3 h, during which time an additional 10 μM/mL of α-factor was added every hour. When ≥90% of the cells appeared unbudded and shmooed, cells were pelleted, washed twice with YPD of normal pH, and resuspended in YPD to a final density of 1 x 10^8 cells/mL. Cells were then either shifted to 37°C immediately or allowed to recover at 23°C for various lengths of time (every 15 min up to 75 min) before shifting to 37°C. Cells were incubated for 3.5 h at 37°C and prepared for cytological examination or for flow cytometry analyses.

Yeast cells were prepared for flow cytometry as described by Hutter & Eipel (1979). The flow cytometer used was a Coulter Epics 753 (Coulter Electronics, Hialeah, FL). The excitation wavelength was 488 nm. A barrier filter was used to remove emissions above 590 nm.

Acknowledgements

We thank Mark Walberg for providing materials for our genetic screen and P. Hieter and F. Spencer, Johns Hopkins University, for the genomic library. We also thank Z. Chen, R. Gibbs and W. Schober for advice. This work was supported by grants from the National Institutes of Health, nos NIH GM44664 to S.J.E. and NIH GM53512 to C.D.A. S.J.E. is a PEW Scholar in the Biomedical Sciences and an Investigator of the Howard Hughes Medical Institute.

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Genes to Cells (1996) 1, 923–942 941


Received: 6 June 1996
Accepted: 11 October 1996