We have isolated a conditional lethal mutant mts3 in the fission yeast Schizosaccharomyces pombe which at the permissive temperature is resistant to the mitotic poison MBC and at the restrictive temperature is defective in metaphase to anaphase transition. The predicted amino acid sequence of mts3 is 36% identical with the budding yeast gene NIN1. NIN1 cloned into a fission yeast expression vector can rescue both mts3 temperature-sensitive and null alleles demonstrating that NIN1 is the budding yeast homologue of the fission yeast mts3 gene. The phenotype of the mts3 null is identical with the mts3 ts mutant demonstrating that the phenotype of the mts3 ts mutant is due to loss of mts3 function. The deduced amino acid sequences of both mts3 and NIN1 show homology to peptidase sequences obtained from subunit 14 of the 26 S protease purified from bovine or human cells.

It is becoming increasingly recognized that the stability of proteins is an important factor in the regulation of gene expression. The main nonlysosomal intracellular proteolysis pathway in the cell is the ubiquitin pathway. The ubiquitin pathway has been implicated in the instability of a number of important regulatory proteins such as p53, c-Myc, c-Mos, and the mitotic cyclins. Ubiquitin is a 76-amino acid protein, that when covalently attached to a lysine residue of a protein, targets it for destruction (reviewed in Refs. 1-3).

Biochemical analysis has determined that a multiprotein complex called the 26 S protease degrades proteins that have been targeted for destruction by the ubiquitin pathway. The 26 S protease is made up of two multiprotein functional components, the catalytic 20 S proteasome and the regulatory complex. The 20 S proteasome is a cylindrical structure composed of 14 different protein subunits and contains all the proteolytic activities of the complex. In vitro analysis on purified 20 S complexes has shown that proteins are degraded in a completely unregulated manner. Regulated proteolysis requires the addition of another multiprotein complex composed of at least another additional 15 different subunits to each end of the 20 S proteasome cylinder to form the 26 S protease. The different subunits of this regulatory complex are named according to their size on an SDS-polyacrylamide gel electrophoresis gel, S1 being the largest and S15 the smallest. With the formation of the 26 S protease, degradation of proteins is now carried out in a highly regulated manner. Certain substrates are degraded only if they have been polyubiquitinated and proteolysis is now ATP-dependent (4, 5).

Recently, we isolated a conditional lethal mutant, mts2, in subunit 4 of the 26 S protease regulatory complex in the fission yeast Schizosaccharomyces pombe. Characterization of the phenotype of mts2 strain at the restrictive temperature demonstrated that the cells arrested at a particular point in the cell cycle, the metaphase stage of mitosis (6). In this paper, we describe another conditional mutant, mts3, which was isolated in the same screen. We show that mts3-1 arrests with a phenotype similar to the mts2 mutant and that the S. cerevisiae NIN1 gene is the budding yeast homologue of the mts3 gene and that both the mts3-1 and NIN1 gene products are similar to subunit 14 of the regulatory complex of the 26 S protease purified from mammalian cells.
Isolation of the Budding Yeast NIN1 Gene—The NIN1 gene was amplified by PCR from S. cerevisiae genomic DNA as a Sall/PstI fragment using the primers NIN1-1’/GCACTGCAGATCCCTCTTTTGC-CCATAG (CGAATTGACTCAC) and NIN1-3’/GCACTGCAGACATATTATTTCCA- TACC (CGAATTGACAC) and the resulting BamHI fragment encoded the mts3 gene with 400 base pairs of its coding sequence deleted. The primers were used: GCAG- GATCCGGGAGCTCAAGTTCTGCTTGCGCATCGTCTGCGCAAGTC and GCAGGATTCCGTTACATTGCGTATGCGG-GTCTTCTAGAGATTACCC. The two primers to the mts3 gene are shown in Fig. 5A, and the other two primers contain sequences 400 base pairs 3’ to the mts3 stop codon and 164 base pairs 5’ of the mts3 gene start codon. The mts3 ura4+ gene was inserted into the BamHI site. The BamHI fragment was cut out, gel-purified, and used to transform a mts3-/mt3 homozygous diploid strain selecting for uracil prototrophy. Stable ura4+ transformants were selected. PCR analysis was used to demonstrate that the ura4+ gene had disrupted the mts3 locus. Primers to the ura4+ gene were used in conjunction with an oligonucleotide to the mts3 genomic sequence just upstream of the original primer site to amplify the mts3 deletion. A fragment of the correct size was generated with DNA prepared from the heterozygous mts3+/mts3 mts3+ura4+ but not from the homozygous mts3+/mts3 diploid (data not shown).

Spore Germination of the mts3+/mts3 ura4+ Diploid—Spore germination was carried out as described in Ref. 16. Sporulation was carried out in malt extract broth at 30°C. Asci formed after 2-3 days of incubation. These asci were then digested with Giysulase (Sigma) overnight at 30°C. When all the vegetative cells had lysed, the spores were harvested by centrifugation, washed once in water, and resuspended in 20 ml of water. The spores were then layered on top of 25 ml of 40% glucose in a 50 ml tube. The tube was spun at 1600 rpm. The pelleted spores were then washed once in water and resuspended in water. The spore concentration was determined by counting on a hemocytometer. 4 × 10^6 spores were inoculated into minimal media supplemented with leucine and adenine and incubated at 20°C for 10 h. The spores were transferred to 32°C and sampled at the times shown.

RESULTS

Isolation of the mts3 Mutant—The mts3 mutant was isolated in a screen to isolate mutants that were both resistant to the microtubule stabilizing drug MBC and also temperature-sensitive for growth. One mutant allele mts3-1 was isolated in the screen. Crossing the mts3-1 mutant strain to wild type cells of the opposite mating type showed that the temperature sensitivity segregated 2:2s in tetrads demonstrating that a single non-mutation was responsible for the conditional lethality. When these segregants were tested for MBC, it was found that MBC cosegregated with the temperature sensitivity demonstrating that the same mutation was responsible for both the MBC and the conditional lethality. Construction of heterozygous mts3+/mts3-1 ade6-M210/ade6-M216 leu1.32/leu1.32 ura4-D18/ura4-D18 h + sporulated diploid strain selecting for ura4 prototrophy. Stable ura4+ transformants were selected. PCR analysis was used to demonstrate that the ura4+ gene had disrupted the mts3 locus. Primers to the ura4+ gene were used in conjunction with an oligonucleotide to the mts3 genomic sequence just upstream of the original primer site to amplify the mts3 deletion. A fragment of the correct size was generated with DNA prepared from the heterozygous mts3+/mts3 mts3+ura4+ but not from the homozygous mts3+/mts3 diploid (data not shown).

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wild type cells are shown in Fig. 2a. After shift to the restrictive temperature, an increasing proportion of the population displayed a characteristic mutant phenotype in that the DNA becomes highly condensed (Fig. 2b, B). Associated with this condensed DNA is a short mitotic spindle (Fig. 2b, A). The condensed DNA is found to be located in the middle of the short spindle (Fig. 2b, C) consistent with these cells being at the metaphase stage of mitosis. After 4 h at the restrictive temperature, up to 75% of cells in the population are displaying this phenotype (Fig. 2c). On cells sampled at later time points, the DNA was never found to separate nor the short spindle elongate to a length typical of anaphase in S. pombe (Fig. 2a, C). The mts3-1 mutant therefore appears to be defective in the metaphase to anaphase transition. This metaphase arrest phenotype was found to be transient in that after further incubation at the restrictive temperature the short metaphase spindle was found to disassemble and the DNA was found to decondense to reform a nucleus of normal appearance. In most cases, the single nucleus became displaced to one end of the cell and a septum formed to divide the cell into an anucleate and nucleate half (Fig. 2b, E). This phenotype is very similar to that shown by the S. pombe mutant mts2-1 isolated in the same screen (6). The only difference between the phenotypes displayed at the restrictive temperature is that a much higher proportion (75%) of the mts3-1 strain showed a metaphase arrest compared to 25% of cells in the mts2-1 culture. The septa that formed appeared aberrant when stained with the septum specific stain calcofluor and did not appear to be functional as cell number was not observed to increase (Fig. 1). Cytoplasmic microtubules were found to re-form after the metaphase spindle disassembled (Fig. 2b, D). These microtubules, however, stained far less efficiently with the anti-tubulin antibody than wild type cells, implying that they could be defective. Interestingly, after extended incubation at the restrictive temperature, the culture did not go through another round of chromosome condensation as has been reported for some of the S. pombe mitotic mutants (17).

Histone H1 kinase assay on an mts3 asynchronous culture shifted from the permissive to the restrictive temperature was performed (Fig. 3A, a) and indicated that H1 kinase activity increases after shift to the restrictive temperature. Quantita-
tion on the PhosphorImager shows that activity peaks after a 2-h incubation at the restrictive temperature displaying approximately 3-fold more activity than cells growing at the permissive temperature. The increase in chromosome condensation in samples taken from the same culture occurs later, at 4 h (Fig. 2c), than the peak in H1 kinase activity. After the peak in activity, the level of H1 kinase activity decreased to a level that was much lower than the original value before shift to the restrictive temperature.

Recently, Schwob et al. (18) showed that the budding yeast SIC1 gene encodes a specific inhibitor of the CLB5/CDC28 kinase and that degradation of SIC1 is required to release the CLB5/CDC28 activity essential for G 1/S phase transition (18). In addition, the degradation of SIC1 protein was shown to be by the ubiquitin pathway. To investigate if a similar inhibition event was occurring in the mts3-1 extracts at the restrictive temperature, mixing experiments were carried out. Increasing amounts of crude extracts prepared from cells sampled 8 h (t = 8 h) after shift to the restrictive temperature were mixed with 50 μg of each extract was separated on an 10% SDS-polyacrylamide gel electrophoresis gel. The samples were transferred to nitrocellulose and probed with an anti-Cdc13 antibody. The antibody was detected with a rabbit monoclonal antibody conjugated to alkaline phosphatase (Promega). The alkaline phosphatase activity was detected according to the manufacturer's instructions.

![Fig. 2. Phenotype of mts3-1 at the restrictive temperature.](image)

A, wild type cells stained with the anti-tubulin antibody TAT1 and DAPI. The two images were merged on the computer to give the image shown. The DAPI signal was changed on the computer from blue to red to increase the resolution of the desired structures. A, interphase cells; B, mitotic metaphase cell; C, late anaphase cell; D, postanaphase. B, mts3-1 cells stained with TAT1 and DAPI after shift to the restrictive temperature. A, B, and C, cells sampled 4 h after shift. A, TAT1 staining of microtubules. B, DAPI staining of the DNA. C, the A and B images were merged on the computer to give the image shown. D and E, cells sampled 7 h after shift. D, cells stained with TAT1 and DAPI. The two images were merged as before to give the image shown. E, cells stained with DAPI and the septum specific stain calcofluor. The septum stains as a band located in the middle of the cell while the DNA is found in the ovoid-shaped nucleus located toward the end of the cell at a location similar to that in D. All the cells in A–E are printed at the same magnification. c, percentage of mts3-1 cells after shift to 36°C that show the condensed DNA phenotype (open squares), the short metaphase mitotic spindle phenotype (open circles), and the septated phenotype (filled circles).

![Fig. 3. Histone kinase activity in the mts3-1 mutant on shift to the restrictive temperature.](image)

A, a, at time 0 h, an early logarithmic growing culture of mts3-1 cells was shifted from 25°C to 36°C sampled at the times shown and assayed for H1 kinase activity. Lane c shows the H1 kinase activity obtained from an exponentially growing haploid wild type culture, b, mixing experiment to assay for possible inhibitor. To 50 μg of extract from t = 0 h, increasing amounts of extract from t = 8 h were added (0, 50, 100, and 150 μg) and then assayed for H1 kinase activity as before. B, steady state levels of Cdc13 protein in mts3-1 after shift to the restrictive temperature. The same extracts assayed in A, panel a, were probed by Western analysis for Cdc13 protein. 50 μg of each extract was separated on an 10% SDS-polyacrylamide gel electrophoresis gel. The samples were transferred to nitrocellulose and probed with an anti-Cdc13 antibody. The antibody was detected with a rabbit monoclonal antibody conjugated to alkaline phosphatase (Promega). The alkaline phosphatase activity was detected according to the manufacturer's instructions.
H1 kinase activity measures the amount of activity of the Cdc2 kinase when it is complexed to the \textit{S. pombe} cyclin B homologue Cdc13. To investigate if the extracts were losing activity due to a decrease in the levels of Cdc13 protein, the extracts assayed in Fig. 3 \textit{A}, were subjected to Western blot analysis with an antibody against the \textit{S. pombe} Cdc13 protein. As shown in Fig. 3 \textit{B}, the level of Cdc13 protein remained essentially constant throughout the course of the experiment. The differences in H1 kinase activity that were observed could be due to differences in the level of the Cdc2 protein or alternatively to differences in post-translational modification of either the Cdc2 or Cdc13 protein.

\textbf{mts3 Mutant Cells Re-replicate Their DNA at the Restrictive Temperature—} An \textit{mts3-1} mutant strain was shifted from the permissive to the restrictive temperature, and cells were sampled for flow cytometry to assay their DNA content. The \textit{mts3} cells replicate their DNA from a 2\text{n} to 4\text{n} content of DNA at 8 h (Fig. 4A). At least two possibilities can explain this re-replication in the \textit{mts3-1} mutant strain at the restrictive temperature. First, \textit{mts3} is involved in the control to ensure that replication occurs only once per cell division cycle. Alternatively, the re-replication observed is an indirect effect due to a breakdown in the normal feedback control caused by the \textit{mts3} cells entering mitosis arresting at metaphase and exiting mitosis (as assayed by loss of H1 kinase activity) before chromosome segregation had occurred. To differentiate between these two possibilities, we crossed the \textit{mts3-1} mutation into a \textit{cdc25} background. \textit{cdc25} cells are temperature-sensitive for growth and at the restrictive temperature the cells arrest in the \text{G2} phase of the cell division cycle before mitosis (8). Therefore, the \textit{mts3cdc25} double mutant would also arrest in \text{G2} before mitosis, and, thus, at the restrictive temperature, no cells should

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{A, FACS analysis of \textit{mts3-1} strain shifted to the restrictive temperature. The top three panels show nitrogen (n) starved wild type haploids which have predominantly cells with a 1\text{n} DNA content (a(i)), exponentially growing wild type haploid which have a 2\text{n} DNA content (a(ii)), and N starved wild type diploid which shows a 2\text{n} and 4\text{n} peak of DNA (a(iii)). Panels b(i), b(ii), and b(iii) show \textit{mts2} cells, panels c(i), c(ii), and c(iii) show \textit{mts3} cells, and panels d(i), d(ii), and d(iii) show wild type cells shifted from 25 °C to 36 °C and sampled at 0 h (i), 4 h (ii), and 8 h (iii) after the shift. For each sample, the 1\text{n}, 2\text{n}, or 4\text{n} peaks obtained are indicated. In b, c, and d, the \textit{mts2}, \textit{mts3}, and wild type strains were all haploid strains growing exponentially prior to the shift. B, FACS analysis of \textit{cdc25mts3} strain shifted to the restrictive temperature. A \textit{cdc25mts3} double mutant strain was shifted to 36 °C, sampled at the times shown, and subjected to FACS analysis. The single 2\text{n} peak of DNA observed is marked.}
\end{figure}
make a mitotic spindle. This would allow the re-replication phenotype to be investigated in the absence of the metaphase arrest phenotype.

At 25 °C, the cdc25mts3 double mutant grew very slowly compared to the cdc25 or mts3 single mutants and appeared elongated, being 2 to 3 times longer at division. At 20 °C, however, the double mutant was observed to grow normally. Consistent with this finding was the observation that cdc25mts3 cells growing at this temperature divided at the roughly same size as wild type cells. Therefore, for the cdc25mts3 double mutant, 20 °C instead of 25 °C was used as the permissive temperature.

An asynchronous culture of cells was shifted from the permissive to the restrictive temperature and sampled at the times shown (Fig. 4B). These cells were then fixed, stained with propidium iodide, and subjected to FACS analysis. During the course of the experiment, at the restrictive temperature, the cells elongated to give the characteristic course of the experiment, at the restrictive temperature, the propidium iodide, and subjected to FACS analysis. During the times shown (Fig. 4B). In conclusion, the observed re-replication in the mts3-1 strain appears to be due to the indirect effect of the cells entering mitosis and not the result of deregulation of the initiation of DNA replication.

Isolation of the mts3 Gene—The mts3-1 gene was isolated by complementation of the temperature-sensitive mutation of the mts3-1 mutant strain using an S. pombe wild type genomic library in the autonomously replicating vector, pSPARS(305). A 3.5-kilobase HindIII fragment was found to rescue the mts3-1 ts defect. This fragment was subcloned into the integrating vector pRS(305) and the 3.5-kilobase fragment used to integrate by homology. The integrant could rescue the temperature-sensitive defect of a mts3-1 strain. The site of integration was mapped relative to the mts3-1 mutation. Out of 19 full tetrads, no temperature-sensitive segregants were observed, indicating that the site of integration was very closely linked to the mts3-1 mutation and therefore the cloned genomic fragment contained the authentic mts3-1 gene and not an extragenic suppressor.

The genomic clone was used to isolate a corresponding cDNA clone carried in the S. pombe expression vector pREP1. Overexpression of the mts3-1 cDNA in pREP1 complemented the ts mutation and resulted in no obvious phenotype. The nucleotide sequence of the mts3-1 cDNA was determined and is shown in Fig. 5A.

Comparison of the mts3-1 coding sequence with other DNA sequences in the EMBL data base revealed a substantial homology with the budding yeast NIN1 gene. The deduced amino acid sequence of mts3-1 is 36% identical with the NIN1 amino acid sequence (Fig. 5B). The nin1 mutant is a conditional lethal mutation in which cells at the restrictive temperature arrest in mitosis with a single nucleus and a G2 DNA content (19). The described phenotype is very similar to that of the mts3-1 strain at the restrictive temperature. In addition, the two proteins are very similar in size.

The S. cerevisiae NIN1 Gene Can Rescue the mts3-1 and Null Alleles—To investigate if the NIN1 gene could be the S. cerevisiae homologue of mts3-1, the NIN1 gene was isolated by PCR from S. cerevisiae genomic DNA and subcloned into the S. pombe expression vector pSP1 to give the plasmid pNIN1 (described under “Experimental Procedures”). This plasmid was then used to transform the mts3-1 strain to investigate if the NIN1 gene could rescue the conditional lethal phenotype of the mts3-1 ts mutation. As shown, the NIN1 gene can rescue, but the vector itself or the mts2-1 cDNA cloned into the same vector
alleles, the mts3+/mts3 null allele combination with the similarity between the deduced amino acid sequences confirms that the NIN1 gene is the budding yeast homologue of the fission yeast mts3+ gene.

Phenotype of the mts3 Null Allele—The mts3+/mts3 null allele combination with the similarity between the deduced amino acid sequences confirms that the NIN1 gene is the budding yeast homologue of the fission yeast mts3+ gene. The mts3+ gene encodes the fission yeast homologue of the S. pombe mts3+ gene.

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This provided genetic evidence for a possible interaction between subunits 4 and 7 in the 26S complex itself. We could find no such genetic evidence for an interaction between the mts2 (subunit 4) and mts3 (subunit 14) gene products either in the phenotype of the mts2mts3 double mutant strain (data not shown) or by rescue of the temperature sensitivity by overexpression of mts2 in a mts3-1 strain or vice versa.

When the mts3′ peptide sequence was compared to other sequences in the EMBL data base, it was found to have 36% identity to the S. cerevisiae gene NIN1. The phenotype of the nin1 mutant at the restrictive temperature is similar to that described for the S. cerevisiae ts mutants cim3(MSS1) and cim5(SUG1) which encode subunits 7 and 8 of the 26S protease (23). We show here that the S. cerevisiae NIN1 gene can rescue both S. pombe mts3 null alleles demonstrating that NIN1 is the budding yeast homologue of the mts3′ gene.

Surprisingly, the 36% identity found between the mts3′ and NIN1 gene products is much lower than that found between the mts2′ and corresponding human S4 homologues, which show 73% identity with each other. Both subunits are present in the same multi-protein complex so what model could account for the difference in divergence between different homologues of one subunit (subunit 4) compared to the other (subunit 14)? Perhaps this could be a consequence of where each protein is situated in the complex itself. Subunit 4, for example, could lie in the middle of the complex in intimate association with a number of different proteins allowing little chance of divergence while subunit 14 could be located at one end of the complex interacting with fewer proteins. Whatever the reason, when homologues of subunit 14 are isolated from other organisms, this reduced degree of identity between homologues should aid in the identification of functionally important domains.
mains critical for subunit 14 activity.

The mts2 and mts3 mutants were both isolated in a screen looking for mutants that were resistant to the mitotic poison MBC. It is not apparent why such a screen should seem to be specific for subunits of the regulatory complex of the 26 S protease. One possible explanation is that the concentrations of MBC used in the screen causes cells to make a slightly defective spindle at the permissive temperature which although it is still able to function relatively normally activates a checkpoint pathway to cause cell cycle arrest. The mutants are defective in this putative checkpoint response and are therefore able to grow at the permissive temperature in the presence of the drug. Whether such a model accounts for the MBCR found in the mutants and whether this is related to mitotic arrest at the restrictive temperature will have to wait until further experiments have been carried out.

The mts2 and mts3 mutants at the restrictive temperature both arrest at the metaphase stage of mitosis. In addition, both genes encode different subunits of the same regulatory complex of the 26 S protease. This implies that some substrate(s) has to be degraded by the ubiquitin pathway to proceed from metaphase to anaphase in mitosis. Recent experiments on frog extracts have essentially come to the same conclusion (24). It was originally thought that cyclin B was one of these substrates and that it had to be degraded for cells to proceed from metaphase to anaphase in mitosis. More recent work has disproved this hypothesis and demonstrated that cyclin B destruction is in fact required for exit from mitosis (24, 25). Recent work on the cyclin B destruction pathway has implied that some components involved in this pathway also seem to be required for the destruction of the substrate(s) to allow metaphase to anaphase transition. ts mutants in three genes were isolated in S. cerevisiae that were defective in the destruction of the budding yeast cyclin B homologue, CLB2. Surprisingly, at the restrictive temperature, all three mutants appear to be defective in metaphase to anaphase transition. When the wild type genes were cloned, all three mutations turned out to be in known genes, CDC16, CDC23, and CSE1. The CDC16 and CDC23 genes encode two members of the TPR family of proteins which are characterized by blocks of 34 amino acid tandem repeats known as tetratricopeptide repeats (26, 27). Antibodies that recognize these proteins and an additional TPR protein called CDC27 were used to show that they are present in a large multiprotein complex purified from Xenopus extracts. This complex was isolated by its ability to attach ubiquitin to cyclin B molecules in a cell cycle-dependent manner, an event which targets the cyclin B molecule for destruction (28–31). To explain the metaphase arrest phenotype in the cdc16 and cdc23 mutants at the restrictive temperature, it is postulated that, in addition to being required for the destruction of cyclin B, they are also required for the destruction of the putative substrate that has to be degraded for metaphase to anaphase transition to occur.

The S. pombe cut9 and nucl2 mutants are temperature-sensitive mutants whose genes encode members of this TPR family (17, 31). nucl2 has the highest degree of similarity to CDC27 while cut9 is most similar to CDC16. At the restrictive temperature, like the mts2-1 and mts3-1 ts strains, both nucl2 and cut9 mutants are defective in metaphase to anaphase transition. Genetic evidence suggests that the nucl2 and cut9 proteins interact with each other (17). The mts2, mts3, nucl2, and cut9 mutants could therefore all define genes required in a pathway in S. pombe to degrade the putative substrate whose destruction has been postulated to be required for sister chromatid separation at the metaphase to anaphase transition in mitosis.

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Addendum—While this manuscript was in the reviewing process, a study of the S. cerevisiae nin1 mutant was published (32). One of the main conclusions was that the NIN1 gene product encoded a subunit of the 26 S regulatory complex consistent with the data published here.

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