The *pad1* Gene Encodes a Subunit of the 26 S Proteasome in Fission Yeast*

(Received for publication, May 29, 1998)

Mary Penney‡, Caroline Wilkinson‡, Mairi Wallace‡, Jean-Paul Javerzat§, Katherine Ferrell¶, Michael Seeger‡, Wolfgang Dubiel¶, Stuart McKay‡, Robin Allshire‡, and Colin Gordon‡

From the ‡MRC Human Genetics Unit Western General Hospital, Edinburgh EH4 2XU Scotland, United Kingdom, §Institut de Biochimie et Genetique cellulaires, 33077 Bordeaux Cedex, France, and ¶Institute of Biochemistry Medical Faculty (Charlie), Humboldt-University, 10117 Berlin, Germany

We have isolated a fission yeast mutant, *mts5-1*, in a screen for mutations that confer both methyl 2-benzimidazolecarbamate resistance (MBCR) and temperature sensitivity (ts) on *Schizosaccharomyces pombe*. This screen has previously isolated mutations in the 26 S proteasome subunits Mts2, Mts3, and Mts4. We show that the mutation in the *mts5-1* strain occurs in the *pad1*+ gene. *pad1*+ was originally isolated on a multicopy plasmid that was capable of conferring staurosporine resistance on a wild type strain. *mts5-1/pad1-1* has a similar phenotype to 26 S proteasome mutants previously isolated in the same screen and we show that Pad1 interacts genetically with two of these subunits, Mts3 and Mts4. In this study we describe the identification of Pad1 as a subunit of the 26 S proteasome in fission yeast.

Ubiquitin-mediated protein degradation is the major nonlysosomal proteolytic pathway. Ubiquitin, a 76-amino acid polypeptide that is highly conserved in eukaryotes, marks proteins for degradation by the 26 S proteasome (1). This system is involved in a wide variety of cellular processes including DNA repair, cell cycle progression, signal transduction, and antigen presentation (2). Ubiquitin-mediated protein degradation is known to account for the turnover of cyclins, cyclin-dependent kinase inhibitors, p53, c-Jun, c-Fos, and other oncproteins (reviewed in Ref. 3).

Although the 26 S proteasome is able to degrade proteins involved in diverse processes, the mechanism is highly specific. Protein degradation is usually brought about by the attachment of polyubiquitin moieties to a lysine residue on the target protein. This acts as a sorting signal targeting conjugated proteins to the 26 S proteasome, which then degrades the polyubiquitinated protein into small peptides (reviewed in Ref. 2 and 4).

The 26 S proteasome consists of two multiprotein complexes, the 20 S core and the 19 S cap. The barrel-shaped 20 S enzymatic core consists of four stacked rings each consisting of seven polypeptides (5). The 19 S regulatory cap complex comprises at least 20 different subunits, which fall into two classes, ATPases and non-ATPases (6). In the presence of ATP, the 19 S cap binds to each end of the 20 S core and confers ATP dependence and specificity for ubiquitinated substrates on the 26 S complex (7). The budding yeast SUG1 gene (8) and the fission yeast *mts2*+ gene (9) are known to encode ATPase components of the 19 S cap, while the fission yeast *mts3*+ and *mts4*+ genes encode non-ATPase subunits (10, 11). At the restrictive temperature the phenotypes of the temperature-sensitive (ts)1 *mts2-1* and *mts3-1* strains are transient cell cycle arrest at metaphase, indicating that the metaphase to anaphase transition is blocked (9, 10). This is similar to ts *sug1-1* mutants that are unable to segregate their DNA and arrest after replication with an anucleate bud at the restrictive temperature (13).

Previously, mutations in the *mts2*+ (9), *mts3*+ (10) and *mts4*+ (11) genes were isolated in a screen for fission yeast mutants that are both resistant to the microtubule destabilizing drug methyl 2-benzimidazolecarbamate (MBCR) and temperature sensitive (ts) for growth. In this communication we describe the cloning and characterization of a fourth mutant identified in the same screen, *mts5-1*. This mutation is rescued by a cDNA clone containing the *pad1*+ gene. *pad1*+ was originally isolated by its ability to confer staurosporine resistance on wild type cells when present on a multicopy plasmid (12). Further analysis showed that the *pad1*+ gene isolated in this screen was truncated, lacking 29 amino acids from the carboxyl-terminal end. Pad1 was originally described as a positive regulator of the *Schizosaccharomyces pombe* transcription factor Pap1 since overexpression of the truncated *pad1*+ gene caused an increase in Pap1-dependent transcription (12). Recently cloning of the human homologue of Pad1, Poh1, was reported (14). Poh1 has been shown to encode a subunit of the human 26 S proteasome. In this communication we demonstrate that Pad1 is a subunit of the 26 S proteasome in fission yeast and that the *mts5-1/pad1-1* strain is defective in the degradation of ubiquitin conjugates. Furthermore we describe genetic interactions between the *mts5*+/*pad1*+ gene and two other genes encoding non-ATPase subunits of the 19 S cap, *mts3*+ and *mts4*+. In addition we have isolated the mouse Pad1 homologue which can rescue the ts phenotype of *mts5-1/pad1-1*, illustrating the highly conserved nature of the 26 S proteasome between eukaryotes.

**MATERIALS AND METHODS**

**Yeast Strains**—The *S. pombe* strains described in this report were all derived from the 972h− and 975h+ wild type heterothallic strains.

---

*This work was supported by Medical Research Council funding (to M. P., C. W., J.-P. J., S. M., R. A., and C. G.), a Boehringer Ingelheim short term fellowship (to M. S.), and a research grant from the Deutsch Forschungsgemeinshaft (to W. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) Y13071.**

**The abbreviations used are:** ts, temperature sensitive; MBCR, methyl benzimidazol-2-yl carbamylate; MBC8, MBC resistance; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescein-activated cell sorting; HA, hemagglutinin.
**RESULTS**

**mts3-1 is Rescued by a cDNA Clone Containing the pad1**

A S. pombe cDNA library was screened to isolate clones capable of rescuing the 

mts3-1 isotype phenotype. One clone was identified and found to contain the pad1 gene and was shown to be the authentic gene by homologous integration (Fig. 1). From now on we will refer to mts5-1 as pad1-1. The mutation in the pad1-1 allele was identified by sequencing. Genomic DNA was isolated from pad1-1 and the pad1 ts allele was amplified by polymerase chain reaction. The mutation was found to be a G to A transition at position 160. This changes the corresponding codon from GGT to AGT, substituting serine for glycine (Fig. 1).

**pad1-1 Arrestrs with the Same Phenotype as the 26 S Proteasome Mutants mts2-1 and mts3-1**—In pad1-1 16% of cells arrest transiently at metaphase with a short mitotic spindle and condensed DNA after 4 h of growth at the restrictive temperature, consistent with a metaphase arrest (Figs. 2 and 3). With further incubation at the restrictive temperature the number of cells at metaphase decreases, while the number of cells with a septated phenotype, elongated septate cell with decondensed DNA in one daughter cell only, increases (Fig. 3). After 24 h of incubation at the restrictive temperature, no metaphase cells are present and 95% of the cells display the septated phenotype. FACS analysis demonstrates that cells arrest with a G2 content of DNA (Fig. 4). This is similar to the 26 S proteasome mutant mts2-1 isolated in the same screen. This mutant arrests transiently at metaphase after 4 h of growth at the restrictive temperature with a G2 content of DNA and forms septated cells with DNA in one daughter cell after extended incubation at the restrictive temperature. These observations coupled with the MBC phenotype, indicated that Pad1 might be a subunit of the 26 S proteasome.

**Pad1 Co-purifies with Known Subunits of the 26 S Proteasome**—To determine whether Pad1 is a subunit of the 26 S proteasome in fission yeast, the 26 S proteasome was purified from wild type S. pombe cells by anion exchange chromatography and glycerol gradient centrifugation as described previously for other fission yeast 26 S proteasome subunits (22). Using anti-HA antibodies against a HA-tagged pad1, Pad1...
was found to co-purify with the fractions containing the highest levels of 26 S proteasome activity. Furthermore, these are the fractions where known subunits of the 26 S proteasome are found (22) (Fig. 5A). Further evidence to indicate that Pad1 is a subunit of the 26 S proteasome in fission yeast was obtained by the finding that it is co-immunoprecipitated with the fission yeast 19 S cap subunit, Mts4 (11). The Mts4 protein was immunoprecipitated from \textit{S. pombe} extracts using antisera raised against the recombinant Mts4 protein (11). Western blots of the immunoprecipitated fractions using an anti-HA monoclonal antibody revealed that the Pad1 protein was also present, as the extracts were made from the strain containing the HA-tagged version of the \textit{pad1} gene (Fig. 5B). As a control, when preimmune sera was used for the immunoprecipitation, neither Mts4 nor Pad1 could be detected.

The \textit{pad1}-1 Mutant Shows an Increase in High Molecular Weight Ubiquitinated Proteins at the Restrictive Temperature—An increase in high molecular weight ubiquitin conjugates has previously been reported in \textit{mts2}-1 after incubation at the restrictive temperature (9). A similar assay was undertaken to ascertain whether \textit{pad1}-1 showed a similar increase, which would suggest it also had a proteolysis defect. Wild type, \textit{mts2}-1 (9), and \textit{pad1}-1 strains were grown overnight at 25 °C to mid log phase, and then shifted to the restrictive temperature of 35 °C. Cells were analyzed at 0, 4, and 8 h after the temperature shift, and proteins were isolated. Equal amounts of protein were run on an SDS-PAGE gel and electroblotted. Blots were probed with either anti-ubiquitin antibodies or anti-actin antibodies to act as a loading control (Fig. 5C). High molecular weight ubiquitinated protein conjugates are present in \textit{pad1}-1 and \textit{mts2}-1 at the permissive temperature, and with incubation at the restrictive temperature of 35 °C these levels increase significantly. A wild type culture grown under identical conditions has no detectable high molecular weight ubiquitinated conjugates at 25 or 35 °C. An increase in high molecular weight
ubiquitin conjugates. These results are consistent with a defect in the degradation of ubiquitin conjugates.

Ubiquitin Conjugate Degradation Is Impaired in pad1-1—Analysis of the degradation of ubiquitin conjugates provides a specific test for 26 S proteasome function (22). After growing cells at the permissive temperature ubiquitin-[125I]-lysozyme conjugate degradation was measured in glycerol gradient fractions possessing the highest activities of the 26 S proteasome. The data are summarized in Table I. The results demonstrate that reduced levels of conjugate degradation are seen in the pad1-1 strain compared with wild type. Residual degradation rates of the pad1-1 26 S proteasomes can be explained by nonspecific cleavage of free lysozyme (22). These results are consistent with pad1-1 having a defect in the degradation of ubiquitin conjugates.

Overexpression of Mts3 and Mts4 Will Rescue pad1-1 at an Intermediate Temperature—The pad1-1 strain will grow at the permissive temperature of 25 °C, but not at the restrictive temperature, 35 °C, or at an intermediate temperature of 32 °C. Plasmids expressing the non-ATPase 19 S cap subunits Mts3 (10) and Mts4 (11) under the control of the thiamine repressible nmt1 promoter were transformed into the pad1-1 strain and streaked onto selective medium with and without thiamine at 25, 30, 32, and 35 °C to test for full or partial rescue of the ts phenotype of pad1-1. Overexpression of Mts3 and Mts4 at 32 °C but not 35 °C will rescue the temperature sensitive phenotype of pad1-1 (Fig. 6). Overexpression of the ATPase 19 S cap subunit Mts2 (9) was unable to rescue the temperature sensitivity of pad1-1 at 32 or 35 °C (data not shown). In addition, pad1-1 was found to be synthetically lethal with mts3-1. Ascii containing four viable, three viable, and two viable spores segregated in a ratio of 4, 18, and 1, respectively. In the tetrads containing three viable spores the missing one was deduced to be the pad1-1mts3-1 double mutant. The same conclusion was drawn from the tetrads containing the two viable spores, which both gave rise to wild type colonies. The double mutant spores were examined microscopically and found to divide once or twice to give a maximum of four cells. An explanation for the partial rescue and the synthetic lethality results is that Pad1 interacts directly with Mts3 in the 19 S regulatory complex. The overexpression data suggests that Pad1 may also interact with Mts4 directly although the mts4-1 and pad1-1 alleles are not synthetically lethal.

A Mouse Pad1 Homologue Rescues the pad1-1 ts Phenotype—pad1+ is essential for cell viability (12). Proteins with highly similar sequences exist in Saccharomyces cerevisiae and humans (Table II). We also isolated a mouse cDNA from a library using the S. pombe pad1+ gene as a probe. The mouse protein shares 68.1% identity with the S. pombe Pad1 protein over its whole length. In addition, the mouse pad1+ gene, when expressed from a S. pombe expression vector, can rescue the temperature sensitivity of pad1-1 (Fig. 7). This demonstrates that the mouse gene is the functional homologue of S. pombe pad1+ gene.

DISCUSSION

In this study we provide biochemical and genetic evidence that the pad1+ gene encodes a subunit of the 19 S regulatory complex of the 26 S proteasome. First, we have shown that the Pad1 protein co-purifies with peak levels of 26 S proteasome activity after anion exchange chromatography followed by glycerol gradient centrifugation. Furthermore, the fractions containing the highest 26 S proteasome activity and Pad1 protein levels were also the fractions in which the Mts4 protein, subunit 2 of the 19 S complex of S. pombe (11) was present. Second, the Pad1 protein was present when the 19 S complex was immunoprecipitated from yeast crude extracts using an antibody against the Mts4 protein (11). Third, we show that polyubiquitin conjugates build up in the pad1-1 mutant strain, as has been shown for other 26 S proteasome mutants (9), and that the pad1-1 mutant strain is defective in ubiquitin-lysozyme conjugate degradation. Fourth, 26 S proteasome function in S. pombe is essential for cell growth, and loss of activity results in a characteristic phenotype, arrest at the metaphase stage of mitosis (9–11). The phenotype of the pad1-1 mutant strain at the restrictive temperature is arrest at the metaphase stage of mitosis consistent with a mutation in a 26 S proteasome subunit. Finally, we have shown a genetic interaction between the pad1-1 strain and the mts3-1 and mts4-1 19 S mutant strains. Taken together the data presented provides compelling evidence that the pad1+ gene encodes a subunit of the 19 S complex. The isolation of the
human pad1 homologue, Poh1, and its identification as a subunit of the human 26 S proteasome confirms our results (14).

The 19 S complex is thought to have at least three biochemical functions; the recognition of polyubiquitinated substrates (23); an isopeptidase activity, which cleaves ubiquitin from the substrate for it to be recycled (24); and an anti-chaperone activity to unfold the substrate protein and present it to the 20 S catalytic complex (25). We have assayed purified Pad1 protein expressed in Escherichia coli for polyubiquitin binding and

![Diagram](image-url)
isopeptidase activity, and preliminary results show no evidence for either activity. The original isolation of pad1 was as a truncated gene on a multicopy plasmid which conferred staurosporine resistance on wild type cells. This can be explained by our identification of Pad1 as a 26 S proteasome subunit. A truncated pad1 gene could produce an abnormal protein that might be expected to disrupt the function of the 26 S proteasome leading to staurosporine resistance, possibly due to decreased proteolysis of a protein required for staurosporine resistance. Such a mechanism could also explain the MBCR observed in the mts2-1, mts3-1, mts4-1, and pad1-1 proteasome mutant strains and why the original genetic screen appears to have enriched for proteasome mutants. Pad1 was originally described as a positive regulator of an AP-1 transcription factor Pap1 since overexpression of Pad1 caused an increase in the levels of Pap1. However, in this communication we have described the identification of Pad1 as a novel subunit of the 26 S proteasome in fission yeast. We postulate that decreased degradation of Pap1 in proteasome mutants results in elevated transcription of genes required for resistance to MBC and staurosporine. Experiments to test this hypothesis are in progress.

We also show that pad1-1 interacts genetically with both mts3-1 and mts4-1. This evidence is based on two observations. First, overexpression of either mts3+ or mts4+ cDNAs will partially rescue pad1-1 lethality at an intermediate temperature. Second, mts3-1 and pad1-1 are synthetically lethal. Since the pad1+, mts3+, and mts4+ genes are not sequence homologues, and therefore presumably do not encode proteins of similar biochemical activities, we hypothesise that the partial rescue of the pad1-1 mutant could be due to stabilization of the proteasome complex.

The original isolation of pad1+ was as a truncated gene on a multicopy plasmid which conferred staurosporine resistance on wild type cells. This can be explained by our identification of Pad1 as a 26 S proteasome subunit. A truncated pad1+ gene could produce an abnormal protein that might be expected to disrupt the function of the 26 S proteasome leading to staurosporine resistance, possibly due to decreased proteolysis of a protein required for staurosporine resistance. Such a mechanism could also explain the MBCR observed in the mts2-1, mts3-1, mts4-1, and pad1-1 proteasome mutant strains and why the original genetic screen appears to have enriched for proteasome mutants. Pad1 was originally described as a positive regulator of an AP-1 transcription factor Pap1 since overexpression of Pad1 caused an increase in the levels of Pap1. However, in this communication we have described the identification of Pad1 as a novel subunit of the 26 S proteasome in fission yeast. We postulate that decreased degradation of Pap1 in proteasome mutants results in elevated transcription of genes required for resistance to MBC and staurosporine. Experiments to test this hypothesis are in progress.

We also show that pad1-1 interacts genetically with both mts3-1 and mts4-1. This evidence is based on two observations. First, overexpression of either mts3+ or mts4+ cDNAs will partially rescue pad1-1 lethality at an intermediate temperature. Second, mts3-1 and pad1-1 are synthetically lethal. Since the pad1+, mts3+, and mts4+ genes are not sequence homologues, and therefore presumably do not encode proteins of similar biochemical activities, we hypothesise that the partial rescue of the pad1-1 mutant could be due to stabilization of the

FIG. 5. A, co-purification of Pad1 with the fission yeast 26 S proteasome complex. Specific peptidase activity, as measured by the cleavage of the fluorogenic peptide succinimidyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, was determined in the presence of ATP for the fractions collected from the glycerol gradient centrifugation. This was performed using pooled fractions from DEAE chromatography that also contained this activity. Western blots of the same fractions, containing 7.5 μg of protein/lane, were probed with either anti-Mts4, anti-20 S, or anti-HA (against Pad1) antisera. The various antisera used in each blot is indicated, and molecular mass markers are shown in kilodaltons. B, co-immunoprecipitation of Mts4 and Pad1. Western blot of immunoprecipitation using preimmune sera (lane 1) and anti-Mts4 antisera. The immunoprecipitated fractions were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with either anti-Mts4 antisera (top panel) or anti-HA monoclonal antisera (bottom panel). Molecular mass markers are shown in kilodaltons. C, an increase in high molecular weight protein conjugates is seen in the temperature-sensitive pad1-1 and mts2-1 26 S proteasome mutants. Western analysis was performed on extracts made from pad1-1, mts2-1, and wild type cells at 25 and 35 °C (4 and 8 h) with an antibodies against ubiquitin (DAKO Z0658) and actin (Amersham N350), to act as a loading control. An increase in high molecular weight protein conjugates is seen in pad1-1 and mts2-1 when incubated at the restrictive temperature, 35 °C. A wild type culture grown under identical conditions contains no high molecular weight ubiquitinated conjugates at 25 °C or 35 °C. pad1-1 and mts2-1 have low levels of ubiquitinated conjugates even at 25 °C indicating that the 26 S proteasome is not fully functional in these mutants even at the permissive temperature.

| Strain at 37 °C | % Ubiquitin degradation per 60 min |
|---------------|---------------------------------|
|               | −ATP                             | +ATP                            |
| Wild type     | 6.38 ± 0.59                      | 10.22 ± 0.61                    |
| pad1-1        | 1.88 ± 0.24                      | 3.58 ± 0.59                     |

2 M. Penney, R. Layfield, and C. Gordon, unpublished data.
heat labile Pad1 protein in the pad1-1 strain. This raises the possibility that the Pad1 protein and the Mts3 and Mts4 proteins interact directly in the 19S regulatory complex.

Previously, a human gene JAB1 which has limited homology to Pad1 over the N-terminal half of the protein (Fig. 1), was isolated (26). As the JAB1 gene could not rescue a pad1 deletion strain, however, it was not a functional homologue (26). The functional Pad1 mouse homologue that we have isolated and the recently described Poh1 human homologue both have greater sequence identity with Pad1 than JAB1 has (Table II) and both are functional homologues of pad1 mutants. Consistent with these observations JAB1 has recently been reported to be a subunit of a high molecular weight complex called the signalosome, distinct from the 26S proteasome and implicated in signal transduction (27). Although JAB1 cannot rescue a pad1 deletion strain, overexpression of JAB1 from a high copy number vector in wild type S. pombe cells was as efficient as pad1 overexpression in conferring drug resistance (26). In addition, overexpression of both JAB1 and S. pombe Pad1 also causes c-Jun-dependent AP-1 transcriptional activity in mammalian cells, leading to the conclusion that Pad1 and JAB1 are co-activators of AP-1 transcription factors (26). In this study however, we have shown that Pad1 is a subunit of the 26 S proteasome in fission yeast, and therefore we offer an alternative mechanism for the above results. Overexpression of JAB1, like overexpression of the truncated Pad1 protein, somehow disrupts 26 S proteasome function and results in elevated levels of AP-1/Pap1, thus leading to drug resistance.

Acknowledgments—We thank Nicholas D. Hastie and Paul Kersey for critical reading of the manuscript and suggestions, Eleanor Simpson, Marina MacKenzie, Siobhan Jordan, Peter Budd, Gordon McGurk, and Kieran J. Jacka for support and encouragement, and Norman Davidson, Sandy Bruce, and Douglas Stuart for excellent photography.

REFERENCES
1. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
2. Jentsch, S., and Schlenker, S. (1995) Cell 82, 881–884
3. Ciechanover, A. (1994) Biol. Chem. 375, 565–581
4. Murray, A. (1995) Cell 81, 149–152
5. Chen, P., and Hochstrasser, M. (1995) EMBO J 14, 2620–2630
6. Dubiel, W., Ferrell, K., and Rechsteiner, M. (1995) Mol. Biol. Rep. 21, 27–34
7. Hochstrasser, M., (1995) Curr. Opin. Cell Biol. 7, 215–223
8. Rubin, D. M., Coux, O., Wedes, I., Hengartner, C., Young, R. A., Goldberg, A. P., and Finley, D. (1996) Nature 379, 655–657
9. Gordon, C., McGurk, G., Wallace, M., and Hastie, N. (1996) J. Biol. Chem. 271, 5704–5711
10. Wilkinson, C. R. M., Wallace, M., Seeger, M., Dubiel, W., and Gordon, C. (1997) J. Biol. Chem. 272, 25768–25777

FIG. 6. Overexpression of cDNAs encoding the 19 S cap subunits Mts3 and Mts4 will rescue the temperature sensitive phenotype of pad1-1 at an intermediate temperature. pad1-1 was transformed with pREP1, mts3+ (pmts3), mts4+ (pmts4), and pad1+ (ppad1) cDNAs under the control of the thiamine repressible promoter in pREP1. Pad1-1 cells containing these plasmids were streaked to single colonies at the permissive (25 °C), restrictive (35 °C), and two intermediate (30 and 32 °C) temperatures.

TABLE II
Comparison of percent identity between full length protein sequences of Pad1 homologues in mouse (mPad1) (Y13071), human Poh1 (86782) (14) and S. cerevisiae (Mpr1) (28)
Lower percent identity of JAB1 (26) to these homologues is also shown.

| Protein sequence | Pad1 | mPad1 | Poh1 | Mpr1 |
|------------------|------|-------|------|------|
| JAB1             | 28.9 | 28.9  | 28.8 | 28.8 |
| Pad1             | 68.1 | 68.1  | 68.1 | 64.5 |
| mPad1            | 99.3 | 99.3  | 99.3 | 65.0 |
| Poh1             |      |       |      | 65.3 |

FIG. 7. The cDNA encoding the mouse homologue of fission yeast Pad1 can rescue the pad1-1 strain at 35 °C. pad1-1 was transformed with the mouse pad1+ cDNA in pREP1 (ppad1+), S. pombe pad1+ cDNA in pREP1 (ppad1+), and pREP1 alone. pad1-1 cells containing these plasmids were then streaked to single colonies at the permissive (25 °C) and restrictive (35 °C) temperatures.
12. Shimanuki, M., Saka, Y., Yanagida, M., and Toda, T. (1995) *J. Cell Sci.* **108**, 569–579
13. Ghislain, M., Udvardy, A., and Mann, C. (1993) *Nature* **366**, 358–362
14. Spataro, V., Toda, T., Craig, R., Seeger, M., Dubiel, W., Harris, A. L., and Norbury, C. (1997) *J. Biol. Chem.* **272**, 30470–30475
15. Moreno, S., Klar, A., and Nurse, P. (1990) *Methods Enzymol.* **194**, 793–823
16. Maundrell, K. (1990) *J. Biol. Chem.* **265**, 10857–10864
17. Maundrell, K. (1993) *Gene (Amst.)* **123**, 127–130
18. Keeney, J. B., and Boeke, J. D. (1994) *Genetics* **136**, 849–856
19. Hagan, I. M., and Hyams, J. S. (1989) *J. Cell Sci.* **89**, 343–357
20. Woods, A., Sherwin, T., Sasse, R., MacRae, T. H., Baines, A. J., and Gull, K. (1989) *J. Cell Sci.* **93**, 491–500
21. Sazer, S., and Sherwood, S. (1990) *J. Cell Sci.* **79**, 509–516
22. Seeger, M., Gordon, C., Ferrell, K., and Dubiel, W. (1996) *J. Mol. Biol.* **263**, 423–431
23. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) *J. Biol. Chem.* **269**, 7059–7061
24. Lam, Y. A., Wu, W., DeMartino, G. N., and Cohen, R. E. (1997) *Nature* **385**, 737–740
25. Rubin, D. M., and Finley, D. (1995) *Curr. Biol.* **5**, 854–858
26. Claret, F.-X., Hibi, M., Dhut, S., Toda, M., and Karin, M. (1996) *Nature* **383**, 453–457
27. Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998) *FASEB J.* **12**, 469–478
28. Rinaldi, T., Bolotin-Fukuhara, M., and Frontali, L. (1995) *Gene (Amst.)* **160**, 135–136