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Regulation of Telomere Length by Checkpoint Genes in *Schizosaccharomyces pombe*

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We have studied telomere length in *Schizosaccharomyces pombe* strains carrying mutations affecting cell cycle checkpoints, DNA repair, and regulation of the Cdc2 protein kinase. Telomere shortening was found in *rad1*, *rad3*, *rad17*, and *rad26* mutants. Telomere lengths in previously characterized *rad1* mutants paralleled the replication checkpoint proficiency of those mutants. In contrast, *rad9*, *chk1*, *hus1*, and *cds1* mutants had intact telomeres. No difference in telomere length was seen in mutants affected in the regulation of Cdc2, whereas some of the DNA repair mutants examined had slightly longer telomeres than did the wild type. Overexpression of the *rad1*$^+$ gene caused telomeres to elongate slightly. The kinetics of telomere shortening was monitored by following telomere length after disruption of the *rad1*$^+$ gene; the rate was ~1 nucleotide per generation. Wild-type telomere length could be restored by reintroduction of the wild-type *rad1*$^+$ gene. Expression of the *Saccharomyces cerevisiae RCK1* protein kinase gene, which suppresses the radiation and hydroxyurea sensitivity of *Sz. pombe* checkpoint mutants, was able to attenuate telomere shortening in *rad1* mutant cells and to increase telomere length in a wild-type background. The functional effects of telomere shortening in *rad1* mutants were assayed by measuring loss of a linear and a circular minichromosome. A minor increase in loss rate was seen with the linear minichromosome, and an even smaller difference compared with wild-type was detected with the circular plasmid.

INTRODUCTION

Checkpoint genes are involved in monitoring the orderly progress of events in the cell cycle and in arresting or delaying cell cycle progress when there is an indication that an earlier process in the cell cycle has not been completed properly. Mutation in checkpoint genes disrupts these dependencies, and in humans it leads to increased sensitivity to DNA-damaging agents and increased susceptibility to cancer, such as in patients with ataxia telangiectasia. Cells from these patients, with mutation of the *ATM* gene, beside a defective checkpoint response to γ-ray–induced DNA damage, also have accelerated shortening of telomeres (Metcalfe *et al*., 1996).

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In *Schizosaccharomyces pombe*, a group of genes has been defined, where a null mutation in any one of the member genes confers simultaneous loss of several checkpoint-related functions. Thus, null alleles of *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, or *hus1*$^+$ lead to cells that no longer have the ability to inhibit mitosis in response to DNA damage in G_2 or to incomplete DNA replication; they also die rapidly in the presence of the DNA synthesis inhibitor hydroxyurea; finally, these mutations interact with *cdc17* or *wee1* mutations (Al-Khodairy and Carr, 1992; Enoch *et al*., 1992; Al-Khodairy *et al*., 1994; Carr and Hoekstra, 1995; Kanter-Smoler *et al*., 1995). It has been possible to partially separate these functions in some mutants. The *rad26-T12* mutant arrests normally in G_2 in response to irradiation but is still moderately radiation sensitive (Al-Khodairy *et al*., 1994); a similar pattern is seen for, e.g. *rad17-E198A* (Griffiths *et al*., 1995). Cells with the

rad1-S3 mutant allele have lost the replication checkpoint while largely retaining G_2 DNA damage checkpoint function; cells with the *rad1-S1* and *rad1-S2* alleles are fully checkpoint proficient, but these alleles confer lethality in a *cdc17* or *wee1* background (Kanter-Smoler *et al*., 1995).

Telomeres are specialized structures at the ends of eukaryotic chromosomes, essential for preventing loss of genetic material on replication of chromosome ends, for preventing chromosome degradation, and for keeping chromosomes from fusing. In vertebrates, as well as in *Sz. pombe* and *Saccharomyces cerevisiae*, the ends of telomeres contain direct repeats of short DNA sequences encoded by the RNA component of the specialized telomere-replicating enzyme, telomerase (Sugawara, 1989; Zakian, 1995). In *S. cerevisiae*, several mutations affecting telomere length are known, e.g. in *TEL1* encoding a putative regulatory protein (a structural homologue of human ATM, of human DNA-dependent protein kinase, and of the *S. cerevisiae* checkpoint protein Mec1), and in the orphan gene *TEL2* (Greenwell *et al*., 1995; Morrow *et al*., 1995; Zakian, 1995; Runge and Zakian, 1996). Hdf1 and Hdf2, the *S. cerevisiae* homologues of the 70- and 80-kDa Ku antigens associated with DNA-dependent protein kinase, respectively, are also required for maintenance of normal telomere length (Boulton and Jackson, 1996; Porter *et al*., 1996). Interestingly, Hdf1 and Hdf2 have been shown to interact with Sir4 (Tsukamoto *et al*., 1997), raising the possibility that Sir4 recruits Hdf1 and Hdf2 to telomeres. Mutation in any of the above genes causes telomeres to be established at a reduced steady-state length. By contrast, *est2* or *tlc1* mutations, resulting in loss of the protein or the RNA component of telomerase, respectively, cause telomeres to shorten progressively. The Cdc13 protein has recently been shown to bind to single-strand DNA at telomeres, and the *cdc13-est* mutation causes progressive telomere shortening similar to the telomerase mutations (Nugent *et al*., 1996). Mutations in the gene encoding the general DNA-binding protein Rap1 cause telomeres to elongate or to shorten, depending on the nature of the mutant allele (reviewed in Zakian, 1995).

In *Sz. pombe*, considerably less is known about the structure of telomeres and of the genetics of telomere regulation. Five of the six telomeric regions in this organism have been cloned and sequenced (Sugawara, 1989). At the terminus, they consist of 200–300 bp with the repeat unit consensus 5′-TTACAG$_{1-8}$-3′ on the G/T-rich (3′ end) strand (Sugawara, 1989; Duffy and Chambers, 1996). It has been demonstrated that these cloned sequences establish a new telomere onto linear DNA molecules (Nimmo *et al*., 1994). The *taz1*$^+$ gene was found to cause telomere shortening when overexpressed and elongation when disrupted (Cooper *et al*., 1997). Recently, the gene encoding the telomerase-catalytic subunit from fission yeast, *trt1*$^+$, was identified (Nakamura *et al*., 1997); *trt1* mutants exhibit progressive telomere shortening similar to that in *S. cerevisiae est2* mutants. In view of the evidence gathered from humans and *S. cerevisiae* suggesting an involvement of certain checkpoint-related genes in regulation of telomere length, we have investigated whether telomere length in *Sz. pombe* is controlled by checkpoint genes.

MATERIALS AND METHODS

Culture Conditions

Vegetative growth under nonselective conditions was in YPD (2% peptone, 1% yeast extract, 2% glucose). For scoring red *ade6* cells, YES (0.5% yeast extract, 3% glucose, 10 μg/ml adenine, and 100 μg/ml each of uracil, leucine, and histidine) was used. YNB (0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% glucose, and 100 μg/ml each of supplements adenine, uracil, leucine, and histidine as appropriate) was the selective medium except in experiments involving expression from the *nmt1* promoter, where instead Edinburgh Minimal Medium (Moreno *et al*., 1991) was used. Cells were grown at 30°C unless noted otherwise. Genetic crosses were done as described (Gutz *et al*., 1974). *Sz. pombe* was transformed by a modified lithium acetate protocol (Kanter-Smoler *et al*., 1994).

Construction of Sz. pombe Strains

PS46 was made by crossing *h*$^+$ *ade6-704 leu1-32 ura4-D18* with *FY759*. The genomic *rad1*$^+$ gene was disrupted in different strains by transformation with *Bam*HI-cut pr1u4 (Sunnerhagen *et al*., 1990); ura$^+$ transformants were selected on basis on their radiation sensitivity, and the correct disruption event was verified by polymerase chain reaction (PCR) performed directly on cells from the colony (Sunnerhagen, 1993). In this manner, the following strains were created: GK20, SPT1, SPT2, SPT3, SPT4, and SPT5 from strain *h*$^+$ *ura4-D18 leu1-32 his3*; PS45 from FY110; and PS47 from PS46. A plasmid containing the *rad1-S56* allele, where the in-frame deletions of *rad1-S5* and *rad1-S6* are combined in one molecule, was constructed as follows. A silent mutation introducing a *Bst*BI site was made at nucleotide 1555 using the mutagenic oligonucleotide 5′-CGGACAATAACGTTCTTCGAAACG-3′ and plasmids pR1S5 and pR1S6 carrying the *rad1-S5* and *rad1-S6* alleles on a 3.4-kb *Bam*HI fragment as templates (Kanter-Smoler *et al*., 1995). The resulting plasmids, pR1S5B2 and pR1S6B2, were both cut with *Bst*BI, and the 1.2-kb *Bst*BI fragment of pR1S5B2 was swapped for the corresponding fragment of pR1S6B2 to create pR1S56B2. Strains GK21 through GK24 carrying mutations *rad1-S3* through *rad1-S6*, and PS56 carrying *rad1-S56* were created by transformation of strain PS36 and selection for 5-fluoroorotic acid resistance as described (Grimm *et al*., 1988; Kanter-Smoler *et al*., 1995). The origins of these and of all other strains are listed in Table 1.

Detection of Telomeres with Southern Blotting

Chromosomal DNA was isolated with a glass bead-phenol extraction protocol (Hoffman and Winston, 1987). To ensure that even amounts of DNA were loaded, agarose gels were photographed after staining with ethidium bromide. As an additional loading control, we used the intensity of a 2.4-kb *Apa*I band of unique *Sz. pombe* genomic DNA (GenBank accession no. U33008) detected with a PCR product generated with the primers 5′-GGATAGTCCGTCACGAATAC-3′ and 5′-GGGATTTCGGGGTCCAGG-3′. Transfer to GeneScreen filters was in 25 mM Na_2HPO_4, pH 6.5. The 1.9-kb *Apa*I fragment of pEN42 (Nimmo *et al*., 1994) was used as a probe for telomeric repeat sequences, and the 0.84-kb *Apa*I fragment of the same plasmid was used as a probe for subtelomeric repeats. Both fragments were labeled with the random hexanucleotide priming

Table 1. *Sz. pombe* strain list

Strain	Genotype	Source or reference
972h^-	h^- (wild-type)	Anwar Nasim
	h^- *ura4-D18 leu1-32 his3*	Paul Russell
	h^+ *ura4-D18 leu1-32 his3*	Paul Russell
	h^- *ura4-D18 leu1-32 ade6-704*	Jürg Kohli
	h^- *rad1-1 ura4*	Anwar Nasim
KLP20	h^- *rad1-S1 ura4-D18 his3 leu1-32*	(Kanter-Smoler *et al*., 1995)
KLP23	h^- *rad1-S2 ura4-D18 his3 leu1-32*	(Kanter-Smoler *et al*., 1995)
GK21	h^- *rad1-S3 ura4-D18 his3 leu1-32*	This work
GK22	h^- *rad1-S4 ura4-D18 his3 leu1-32*	This work
GK23	h^- *rad1-S5 ura4-D18 his3 leu1-32*	This work
GK24	h^- *rad1-S6 ura4-D18 his3 leu1-32*	This work
PS56	h^- *rad1-S56 ura4-D18 his3 leu1-32*	This work
PS36	h^- *rad1::ura4*$^+$ *ura4-D18 his3 leu1-32*	(Dahlkvist *et al*., 1995)
PS32r	h^- *rad1::ura4*$^+$ *ura4-D18 ade6 leu1*	(Sunnerhagen *et al*., 1990)
GK20	h^+ *rad1::ura4*$^+$ *ura4-D18 his3 leu1-32*	This work
	rad3-136 ura4 leu1	Anwar Nasim
NRC2341	h^- *rad3-136 ade7*	Anwar Nasim
NRC3239	*rad5 ura4*	Anwar Nasim
NRC3242	h^- *rad8-190 ura4*	Anwar Nasim
	rad9-192 ade6 ura4	Anwar Nasim
NRC3241	h^- *rad13 ade6 ura4*	Anwar Nasim
NRC3240	h^- *rad16 ade6 ura4*	Anwar Nasim
	rad17-W	Anwar Nasim
GK3	*rad17-W his3 ura4 leu1*	(Dahlkvist *et al*., 1995)
	rad21-45 ura4 leu1	Anwar Nasim
	h^- *rad26::ura4*$^+$ *leu1-32 ade6-704*	Anthony Carr
	h^- *chk1::ura4*$^+$ *leu1-32 ade6-704*	Anthony Carr
	hus1::LEU2	Anthony Carr
	cds1::ura4$^+$	Hiroto Okayama
	cdc2-3w ura4 leu1 his3	Paul Russell
PR 87.97	h^- *cdc2-1w leu1 ura4-D18*	Paul Russell
cdc25OP	*adh:cdc25*$^+$ *leu1-32*	Paul Russell
PGYQ686	*wee1::ura4*$^+$	Paul Young
AD1	h^- *ura4::nmt1:RCK1*	(Dahlkvist *et al*., 1995)
AD2	h^- *rad1::ura4::nmt1:RCK1 ura4-D18 his3 leu1-32*	(Dahlkvist *et al*., 1995)
FY110	h^+ *ura4-D18 leu1-32 ade6-M210* [Ch16 *ade6-M216*]	(Niwa *et al*., 1989)
FY759	h^- *ade6-704* [CM3112 *sup3-5*]	(Allshire *et al*., 1995)
PS45	h^+ *rad1::ura4*$^+$ *ura4-D18 leu1-32 ade6-M210* [Ch16 *ade6-M216*]	This work
PS46	*ura4-D18 leu1-32 ade6-704* [CM3112 *sup3-5*]	This work
PS47	*rad1::ura4*$^+$ *ura4-D18 leu1-32 ade6-704* [CM3112 *sup3-5*]	This work
SPT1	h^+ *ura4-D18 leu1-32 his3 rad1::ura4*$^+$ [pIRT2R1 *radl*$^+$ *LEU2*]	This work
SPT2	h^+ *ura4-D18 leu1-32 his3* [pGSR3 *nmt1:radl*$^+$ *LEU2*]	This work
SPT3	h^+ *ura4-D18 leu1-32 his3 rad1::ura4*$^+$ [pIRT2 *LEU2*]	This work
SPT4	h^+ *ura4-D18 leu1-32 his3 rad1::ura4*$^+$ [pIRT2RCK1 *RCK1 LEU2*]	This work
SPT5	h^+ *ura4-D18 leu1-32 his3 rad1::ura4*$^+$ [pIRT2RCK2 *RCK2 LEU2*]	This work

method (Feinberg and Vogelstein, 1983). Alternatively, the 1.9-kb fragment was labeled by extension of the telomere-specific primer 5′-CCCTGTAA-3′. Conditions for this labeling method were as for random priming, except 100 ng of primer were used. Hybridization was at 58°C in 1% bovine serum albumin; 1 mM EDTA; 0.5 M $NaHPO_4$, pH 7.2; 7% SDS. Filters were washed at 58°C with 0.2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate); 0.5% SDS.

Minichromosome Mitotic Stability Test

Strain FY110 and its *rad1* derivative PS45, carrying the linear minichromosome Ch16 (Niwa *et al*., 1989) was used to assess fidelity of mitotic chromosomal transmission. Ch16 carries the *ade6-M216* allele, and so in a *ade6-M210* chromosomal background cells having lost it are detected by their red color on plates with low adenine concentration. The circular minichromosome CM3112 in PS46 and its *rad1* derivative PS47, contains *sup3-5*, which suppresses the chromosomal *ade6-704* allele and gives an ade$^+$ phenotype. Loss of CM3112 likewise yields an *ade6* phenotype, scored by red color on low adenine plates. The frequency of minichromosome loss per generation was calculated directly from the fractions of half-sectored and quarter-sectored colonies (Allshire *et al*., 1995).

RESULTS

Telomeres Are Shorter Only in Mutants Affected in the Replication Checkpoint

A number of *Sz. pombe* wild-type and mutant strains were subcultured in liquid medium for 100 genera-

tions. Then, chromosomal DNA was prepared and analyzed for the length of telomeres by Southern blot. The rationale for first subculturing for this number of generations was to ensure that each strain had experienced at least 100 divisions since the time the relevant mutation was introduced. This is based on previous experience from *Saccharomyces cerevisiae*, where 100–150 generations of growth after introduction of the *tel1* and *tel2* mutations were required for telomeres to reach their shortest length, a phenomenon called phenotypic lag (Lustig and Petes, 1986). The outcome is shown in Figure 1. In the lower part of the blot, the characteristic smear of telomeric DNA is seen; in addition, several higher molecular weight bands are seen corresponding to telomere-associated repeats (TAR), internal to the telomere. This is as expected as the 1.9-kb *Apa*I fragment of pEN42 used as a probe contains both telomeric repeats and telomere-associated sequences and since telomeric repeat sequences are found also at internal locations near the telomere (Sugawara, 1989; Nimmo *et al*., 1994). There are considerable strain-to-strain variations in number, intensity, and size of the TAR bands (Figure 1A). This is seen even in strains with a lineage that had split very recently, such as in Figure 2, lanes 2 through 9. Because we have not been able to correlate these variations with either type of mutation or state of the telomeres proper, we conclude that TARs are subject to rapid and apparently stochastic rearrangements. For the remainder of this work, we will deal only with the telomeric repeats.

As seen in Figure 1A, there is a clear reduction in telomere length in *rad1*, *rad3*, *rad17*, and *rad26* strains (lanes 2, 3, 4, 6, and 7), most markedly in *rad3* and *rad26* mutants. To eliminate the possibility that the short telomeres found in these strains were merely the fortuitous result of some cryptic mutation, we examined several strains with mutations in the above genes. Two different *rad3* strains (Figure 1A, lanes 3 and 4) had shorter telomeres; further, a *rad1-1* strain as well as several Δ*rad1* strains resulting from independent disruption events all had telomeres shortened to approximately the same length (Figure 1A, lane 2; Figure 2, lanes 11–13).

All of these mutants belong to the "checkpoint-rad" group, the members of which are deficient in both the DNA replication and the G_2 DNA damage checkpoints (Al-Khodairy *et al*., 1994; Carr and Hoekstra, 1995). However, this group also includes the *rad9* and *hus1* mutants, which have intact telomeres (Figure 1, A, lane 5, and B, lane 3). The *cds1* mutation has the distinction of eliminating only the DNA replication checkpoint, while leaving the G_2 DNA damage checkpoint response intact (Murakami and Okayama, 1995). The *chk1* mutation causes deficiency in the G_2 DNA damage checkpoint but does not affect the replication checkpoint (Walworth *et al*., 1993); thus, it has the

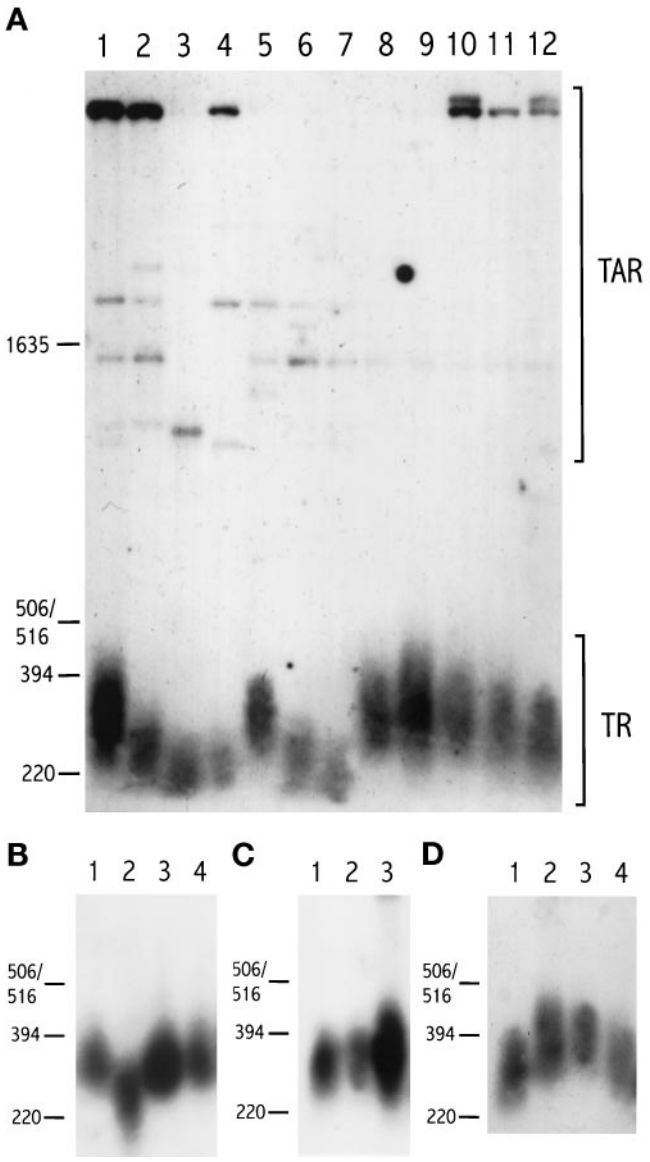

Figure 1. Southern blot analysis of telomere length in different mutant and wild-type *Sz. pombe* strains. Cells were grown for ~100 generations before harvest. Genomic DNA was restricted with *Apa*I and separated on 1.2% agarose gels. DNA was blotted to filters and probed with the 1.9-kb *Apa*I fragment of pEN42 (Nimmo *et al*., 1994). Positions of DNA size markers and their sizes in base pairs are given on the left. (A) Lane 1, strain 972h^- (wild type); lane 2, h^- *rad1-1 ura4*; lane 3, *rad3-136 ura4 leu1*; lane 4, NRC2341 (h^-*rad3-136*); lane 5, *rad9-192 ade6 ura4*; lane 6, GK3 (*rad17*); lane 7, h^- *rad26::ura4*$^+$ *leu1-32 ade6-704*; lane 8, h^- *chk1::ura4*$^+$ *leu1-32 ade6-704*; lane 9, PR 87.97 (*cdc2-1w*); lane 10, *cdc2-3w ura4 leu1 his3*; lane 11, PGYQ686 (*wee1::ura4*$^+$); lane 12, cdc25OP (*adh:cdc25*$^+$). Brackets indicate the position of internal TAR and of the heterogeneous telomeric restriction fragment (TR). (B) Lane 1, 972h^-; lane 2, h^- *rad26::ura4*$^+$; lane 3, *hus1::LEU2*; lane 4, *cds1::ura4*$^+$. (C) Lane 1, 972h^-; lane 2, NRC3242 (*rad8*-190); lane 3, NRC3239 (*rad5*). (The amount of DNA loaded in lane 3 is greater than in the other two lanes.) (D) Lane 1, 972h^-; lane 2, NRC3241 (*rad13*); lane 3, NRC3240 (*rad16*); lane 4, *rad21-45 ura4 leu1*.

complementary effect of *cds1*. These two latter checkpoint mutants also have telomeres of wild-type length (Figure 1, A, lane 8, and B, lane 4).

Mutants affected in other functions were also examined for telomere length, e.g. strains with alterations in genes the products of which affect the activity of the Cdc2/Cdc13 mitosis-promoting factor kinase. Strain PGYQ686 lacks the Wee1 protein kinase, altering the balance toward the Tyr15 unphosphorylated, active form of Cdc2 (Russell and Nurse, 1987). Strain cdc25OP overproduces the Cdc25 protein phosphatase, giving the same effect (Russell and Nurse, 1986). Cells carrying *cdc2-1w* or *cdc2-3w* are smaller at division because of altered sensitivity of the mutant Cdc2 to inhibitory Tyr15 phosphorylation (Russell and Nurse, 1987). None of these strains have significant alterations of telomere length (Figure 1A, lanes 9 through 12). We further examined *rad5*, *rad8*, *rad13*, *rad16*, and *rad21*, which are all deficient in various aspects of DNA repair (Birkenbihl and Subramani, 1992; Doe *et al*., 1993; Carr *et al*., 1994; Lehmann, 1996). None of these strains have shorter telomeres (Figure 1, C and D). On the contrary, some mutants with DNA repair defects have somewhat longer telomeres. This was most clearly seen for *rad13* and *rad16* cells. A marginally increased length was found in *rad5* cells, whereas *rad8* and *rad21* strains had telomeres of wild-type length.

Telomere Length in Wild-Type and Checkpoint Mutant Cells

An estimate of the distance from the terminal *Apa*I site to the start of telomeric repeat sequences on the *Sz. pombe* chromosome ends (Sugawara, 1989) indicates an average of 50 bp. Given this value, we calculate that in 972h^- wild-type cells, the mean length of the telomeric repeat region is about 250 bp (e.g. lane 1 of all panels of Figure 1). This figure is in agreement with several earlier estimates of telomere length in wild-type *Sz. pombe* (as discussed in Funabiki *et al*., 1993). In the four mutant strains with shortened telomeres, the corresponding value is 190 bp for *rad1* and *rad17* mutants and 170 bp for *rad3* and *rad26* mutants (Figure 1A, lanes 2–4, 6, and 7; Figure 1B, lane 2). In two other wild-type strains, h^- *ura4-D18 leu1-32 his3* and h^+ *ura4-D18 leu1-32 his3*, telomeres were slightly longer than in 972h^- or ~275 bp (Figure 2, lanes 2, 10, and 14). Nevertheless, *rad1* strains directly derived from these strains (PS36, GK20) by genomic disruption have telomeres as short as those of *rad1* strains of other origins (*rad1-1*, PS32r) (Figure 1A, lane 2; Figure 2, lanes 11–13). This size reduction is roughly comparable to that calculated for *S. cerevisiae tel2* mutants that carry terminal poly$(TG)_{1–3}$ tracts of ~190 bp and the isogenic wild-type strain 360-bp tracts (Lustig and Petes, 1986).

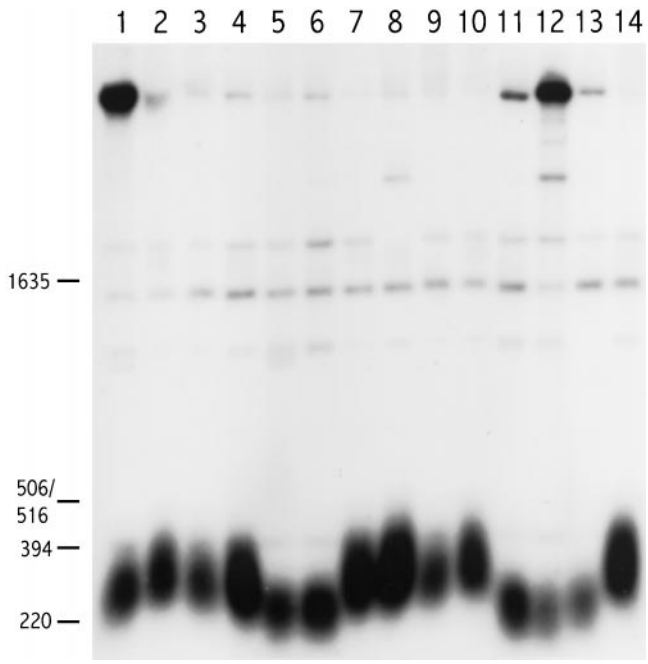

Figure 2. Telomere lengths in different *rad1* mutants. Cells were grown for ~100 generations, and DNA was analyzed as described in the legend to Figure 1. Lane 1, 972h^-; lane 2, h^- *ura4-D18 leu1-32 his3*; lane 3, KLP20 (*rad1-S1*); lane 4, KLP23 (*rad1-S2*); lane 5, GK21 (*rad1-S3*); lane 6, GK22 (*rad1-S4*); lane 7, GK23 (*rad1-S5*); lane 8, GK24 (*rad1-S6*); lane 9, PS56 (*rad1-S56*); lane 10, h^+ *ura4-D18 leu1-32 his3*; lane 11, PS36 (*rad1::ura4*$^+$); lane 12, PS32r (*rad1::ura4*$^+$); lane 13, GK20 (*rad1::ura4*$^+$); lane 14, h^+ *ura4-D18 leu1-32 his3*.

Telomere Length among rad1 Mutants Correlates with Their Replication Checkpoint Proficiency

Having shown that mutation in each of the genes examined with a role in the replication checkpoint cause shorter telomeres, we next examined the influence of different site-specific mutations in the *rad1*$^+$ gene on telomere length. Previously, we have described site-specific *rad1* mutations that affect the various aspects of the *rad1* null phenotypes differently (Kanter-Smoler *et al*., 1995). Strains carrying mutations *rad1-S1* through *-S6*, and *rad1-S56* (Table 1) were grown as above and analyzed for telomere length (Figure 2). Five of the mutants, *rad1-S1, -S2, -S5, -S6,* and *-S56*, had wild-type telomeres, whereas the remaining two, *rad1-S3* and *S4*, had telomeres as short as strains carrying the *rad1::ura4*$^+$ null allele. This was as expected for four of the alleles, since *rad1-S4* is equivalent to the null allele in all aspects examined thus far; *rad1-S5* and *-S6* (Kanter-Smoler *et al*., 1995) and *rad1-S56* (data not shown) perform at least as well as the wild-type allele in all other functions studied. The outcome for the other three alleles makes it possible to correlate telomere shortening specifically with one of the phenotypes of the *rad1* null allele. Mutants carrying *rad1-S1* or *-S2* are proficient with respect to both

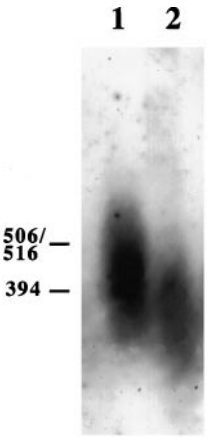

Figure 3. Telomere length after overexpression of *rad1*$^+$. Cells were grown for 100 generations and Southern blot analysis was as detailed in the legend to Figure 1. Lane 1, SPT2 (wild-type strain *h*$^+$ *ura4-D18 leu1-32 his3* transformed with pGSR3 carrying *rad1*$^+$ cDNA under control of the *nmt1* promoter); lane 2, *h*$^+$ *ura4-D18 leu1-32 his3*.

the DNA replication and the G_2 DNA damage checkpoint (Kanter-Smoler *et al*., 1995); these mutants have wild-type telomeres. By contrast, the *rad1-S3* mutation completely abolishes the replication checkpoint, while leaving the G_2 DNA damage checkpoint largely intact (Kanter-Smoler *et al*., 1995); such mutants have telomeres as short as those of *rad1* null mutants. Thus, in this set of mutants, the presence of the replication checkpoint yields telomeres of wild-type length, whereas its absence causes telomeres to shorten.

Overexpression of Rad1 Lengthens Telomeres

Plasmid pGSR3 (Long *et al*., 1994), containing full-length *rad1*$^+$ cDNA under control of the *nmt1* promoter, was used to transform *h*$^+$ *ura4-D18 leu1--32 his3* to leu$^+$. Transformants were cultured for ~100 generations before harvest, allowing full expression from the *nmt1* promoter. As seen in Figure 3, cells overexpressing Rad1 have slightly longer telomeres than their wild-type progenitors.

Expression of S. cerevisiae Rck1 Gives Longer Telomeres

The *RCK1* and *RCK2* genes from *S. cerevisiae* were isolated as suppressors of radiation and hydroxyurea sensitivity of *Sz. pombe* checkpoint mutants (Dahlkvist *et al*., 1995). We wanted to see whether they would also suppress this novel checkpoint mutant phenotype. First, a wild-type strain was transformed with either *RCK1* or *RCK2* cloned in the multicopy vector pIRT2, or with vector only. Then, the *rad1*$^+$ gene was disrupted by transformation with pr1 u4 (Sunnerhagen *et al*., 1990). DNA was harvested after ~100 generations of growth postdisruption. Under this regimen, telomere shortening was only marginally counteracted by expression of Rck1 or Rck2 (data not shown). Second, strains AD1, containing a chromosomally integrated copy of the *S. cerevisiae RCK1* gene under control of the *nmt1* promoter, and AD2, containing the same construct in a Δ*rad1* background, were cultured for 100 generations under conditions allowing maximal expression from the *nmt1* promoter (liquid medium lacking thiamine). Figure 4 shows that cells expressing high levels of *RCK1* have longer telomeres than their counterparts not expressing this gene. AD1 (Figure 4, lane 1) was constructed from the wild-type strain *972h*$^-$ (lane 2), and has telomeres clearly longer than those of *972h*$^-$. AD2 expressing *RCK1* (lane 3) was constructed from the *rad1::ura4*$^+$strain PS36 (lane 4) and has longer telomeres than PS36, although not as long as in the wild-type case.

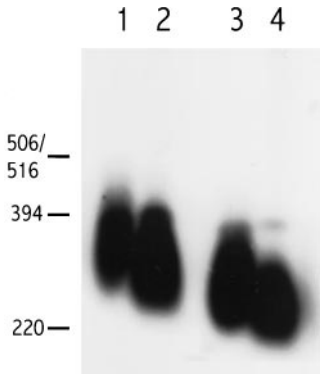

Figure 4. Expression of the *S. cerevisiae* protein kinase-encoding gene *RCK1* from the regulatable *nmt1* promoter in a chromosomally integrated construct causes telomere elongation in *Sz. pombe*. Southern blot analysis as detailed in the legend to Figure 1. Lane 1, AD1 (*972h*$^-$-derived strain expressing *RCK1*); lane 2, *972h*$^-$; lane 3, AD2 (strain expressing *RCK1*; derived from PS36); lane 4, PS36 (*rad1::ura4*$^+$).

Kinetics of Telomere Shortening on Depletion of Rad1

In an experiment to measure the time after inactivation of the *rad1*$^+$ gene required for telomeres to shorten below their normal length, the chromosomal *rad1*$^+$ gene was disrupted as above in a wild-type background (*h*$^+$ *ura4-D18 leu1-32 his3*). In Figure 5, the telomere lengths of these freshly generated disruptants at various time intervals are seen and compared with the wild-type progenitor strain. For the first time point, a colony of disruptants was expanded in liquid medium until the total number of progeny cells was 5×10^8; this is equivalent to ~30 cell divisions since the original gene disruption event, assuming cell death to be insignificant. There is a gradual decrease of average telomere size up to the last time point taken (lanes 1–4).

Shortening of Telomeres after Rad1 Depletion Is Reversible

Having followed the approximate time course of telomere shortening, we then wanted to investigate

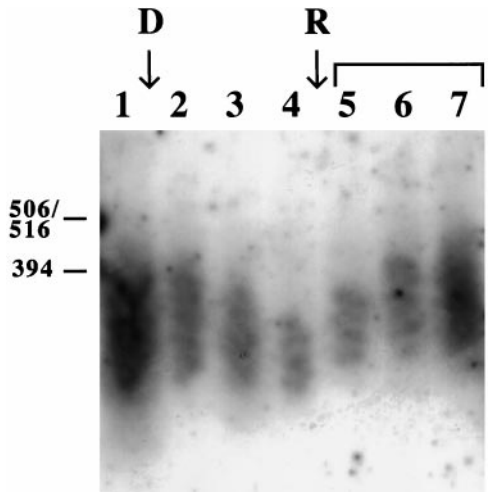

Figure 5. Time course of telomere shortening after disruption of the *rad1*$^+$ gene and of restoration of wild-type telomeres after reintroduction of wild-type *rad1*$^+$. Wild-type strain *h*$^+$ *ura4-D18 leu1-32 his3* was transformed with pr1u4 and DNA was isolated at various times thereafter. D, point where the *rad1*$^+$ gene was disrupted; R, point where the *rad1*$^+$ gene was reintroduced. Brackets above lanes indicate where the *rad1*$^+$ gene was expressed from a multicopy plasmid (pIRT2R1). Southern blot as described in the legend to Figure 1. Lane 1, DNA was isolated before disruption (0 h); lane 2, 30 generations postdisruption (transformant colony expanded to 5×10^8 cells); lane 3, 60 generations; lane 4, 100 generations postdisruption. The disruptant strain was then transformed with plasmid pIRT2R1 containing the wild-type *rad1*$^+$ gene. Lane 5, 30 generations after reintroduction of *rad1*$^+$; lane 6, 50 generations; lane 7, 100 generations.

whether telomere length could be restored by reintroducing the *rad1*$^+$ gene. The Δ*rad1* disruptant strain was transformed with pIRT2R1, which carries the wild-type *rad1*$^+$ gene on a multicopy vector.

Again a transformant colony was expanded, and DNA was prepared 30 generations posttransformation. As seen in Figure 5, lanes 5 through 7, after reexpression of *rad1*$^+$ telomeres gradually grow longer, and after 100 generations they have regained their original size.

Chromosome Stability in rad1 Mutants

The *rad1*$^+$ gene was disrupted in strain FY110, which carries the linear 0.5-Mb minichromosome Ch16, to yield strain PS45. This isogenic pair of strains was grown for 100 generations in liquid medium lacking adenine, thus selecting for the minichromosome. As seen in Figure 6A, there is an ~2-fold increase in loss rate of the linear minichromosome in a *rad1* background. In an analogous experiment, the loss rate of the 30-kb circular minichromosome CM3112 was estimated in an isogenic *rad*$^+$*-rad1* pair (PS46 and PS47) after 100 generations of growth following the gene disruption event. In this case the increase in loss rate in the *rad1* strain was less, or about 1.5-fold (Figure 6B).

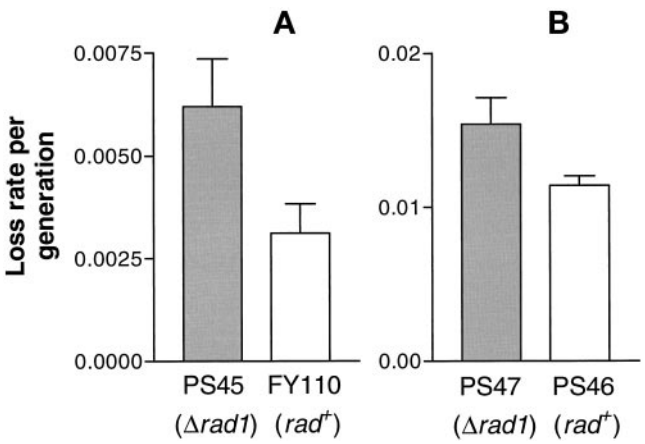

Figure 6. Frequency of minichromosome loss in wild-type and Δ*rad1* strains. The genomic *rad1*$^+$ gene was disrupted in a strain carrying the minichromosome, and the resultant strain and its wild-type parent were grown for ~100 generations. The frequency of chromosome loss per generation was estimated directly from the fraction of half-sectored colonies plus one-half the fraction of colonies with one-quarter section red and three-quarters white. Each value given is the average of at least three readings and represents counting of at least 5000 colonies. Error bars, ± 1 SE of the mean. (A) Linear minichromosome Ch16; (B) circular minichromosome CM3112.

Temperature-sensitive Slow Growth

The *S. cerevisiae tel1* and *tel2* mutants exhibit slow growth at 37°C, but only after the phenotypic lag of ~100 generations (Zakian, 1995; Runge and Zakian,

Table 2. Semiquantitative test of growth at elevated temperature (37°C) of selected mutants, after subculturing for 100 generations

Strain	Growth at 37°C
h$^-$ *ura4-D18 leu1-32 his3* (wild-type)	+++
PS32r (Δ*rad1*)	+++
KLP20 (*rad1-S1*)	+++
GK21 (*rad1-S3*)	+++
GK22 (*rad1-S4*)	+++
PS56 (*rad1-S56*)	+++
rad3-136 ura4 leu1	+
NRC234 (*rad3-136*)	+
NRC3239 (*rad5*)	+++
NRC3242 (*h*$^-$ *rad8-190*)	+++
rad17-W	+++
rad21-45 ura4 leu1	+
h$^-$ *rad26::ura4*$^+$ *leu1-32 ade6-704*	++
h$^-$ *chk1::ura4*$^+$ *leu1-32 ade6-704*	+++
hus1::LEU2	++
cds1::ura4$^+$	+++
PGYQ686 (Δ*wee1*)	+

Cells were deposited as liquid droplets on solid YPD medium and grown at 30°C or 37°C for 2 days. +++, growth at 37°C unaffected relative to 30°C; ++, slightly retarded growth at 37°C; +, markedly retarded growth.

1996). We investigated the growth rate at 37°C of selected mutants after 100 generations of subculturing in liquid medium. As seen in Table 2, such slow growth was found in *rad3*, *rad21*, *rad26*, *hus1*, and *wee1* strains. All other strains examined, including *rad1* and *rad17* strains, grew as fast as wild-type strains at this temperature.

DISCUSSION

We have established short telomeres as a novel phenotype of mutants of four of the *Sz. pombe* checkpoint genes. Mutations in the human *ATM* gene cause cell cycle checkpoint defects as well as telomere shortening (Savitsky *et al*., 1995; Metcalfe *et al*., 1996). In *S. cerevisiae*, there is a separation of these phenotypes such that *mec1* mutants have checkpoint deficiencies but normal telomeres, whereas *tel1* mutants have shortened telomeres but no checkpoint defect (Weinert *et al*., 1994; Greenwell *et al*., 1995; Morrow *et al*., 1995; Paulovich and Hartwell, 1995). Mutation in the structurally related *TOR1* and *TOR2* genes does not affect telomere length (Greenwell *et al*., 1995). The situation in *Sz. pombe* in this respect is similar to the human one in that mutation in the homologous gene *rad3*$^+$ causes both checkpoint and telomere defects.

Among the mutants studied in this paper, we found shortening of telomeres exclusively in cell cycle checkpoint mutants. We studied *rad1* mutants in more detail and found telomere shortening among these to correlate with loss of the replication checkpoint. However, there must be additional criteria since *rad9*, *hus1*, and *cds1* mutants, all of which lack the replication checkpoint, have normal telomeres. For *hus1* and *cds1*, null mutants were used. For *rad9*, the *rad9*-192 point mutation was studied; however, this allele is indistinguishable from the null allele with respect to UV and γ resistance (Murray *et al*., 1991). Assuming that the six "checkpoint-rad" gene products act as a multiprotein complex (Al-Khodairy *et al*., 1994; Carr and Hoekstra, 1995) when instigating the cell cycle responses to DNA damage or unreplicated DNA, then a related complex may exist with only four members (Rad1, Rad3, Rad17, and Rad26) with a role in control of telomere length.

We found no reduction of telomere length in mutants affected directly in various aspects of regulation of Tyr15 of the Cdc2 protein. This is noteworthy given that *chk1*$^+$-dependent signalling from checkpoint proteins has been shown to go through the protein phosphatase Cdc25, responsible for dephosphorylating this residue (Furnari *et al*., 1997). All the mutants in this group tested here (*cdc2-1w*, *cdc2-3w*, *adh:cdc25*$^+$, Δ*wee1*) have alterations that up-regulate Cdc2 activity and would be predicted to have shorter telomeres if defective Cdc2 Tyr15 signalling were the cause of the short telomeres in checkpoint mutants. Because this is clearly not the case, the signalling from checkpoint proteins which is relevant for telomere length control may be separate from the one involving phosphorylation of Cdc2 Tyr15 and cell cycle arrest.

Among DNA repair mutants, three were found to have slightly longer telomeres than the wild type. These, *rad5*, *rad13*, and *rad16* are all defective in nucleotide excision repair (NER). We can only speculate that several components of the NER machinery are required for normal function of telomere processing and maintenance. *rad8* and *rad21* mutants have normal telomeres; *rad8*$^+$ is implicated in an uncharacterized pathway separate from NER, whereas the *rad21* mutation is thought to confer a defect in double-strand break repair (Lehmann, 1996).

More than one mechanism for telomere shortening has been demonstrated in *S. cerevisiae*. Loss of DNA corresponding to the length of an RNA primer during replication of the 5′ end of one of the strands predicts a rate of ~10 bp per generation, uniform for all individual chromosome ends. Telomeric rapid deletion (Li and Lustig, 1996) can shorten an individual telomere by >1 kb in a single cell division; however, this process affects only a small subset of the population each generation. After eliminating *rad1*$^+$ expression, *Sz. pombe* telomeres gradually shorten with a rate of ~1 nucleotide per generation. This does not contradict a model in which telomeres are shortened for every round of replication. The length of telomeres in replication checkpoint mutants with an undetermined number of generations of growth prior to subculturing for 100 generations in this work (*rad1-1*, *rad17-W*) was the same as in those disrupted in *rad1* during the course of this work and then subcultured for 100 generations. This indicates that in these mutants, a new equilibrium is reached, different from that in wild-type cells. Thus, this type of telomere shortening is analogous to what is found in, e.g., *S. cerevisiae tel1* and *tel2* mutants, but distinct from the ever-shorter phenotype of *S. cerevisiae est2* or *tlc1* mutants. The short telomeres of *S. cerevisiae tel2* mutants revert to wild-type length ~50 generations after introduction of the *TEL2* gene (Runge and Zakian, 1996). The rate of the corresponding reversion in *Sz. pombe rad1* mutants on reexpression of the *rad1*$^+$ gene (wild-type length after 100 generations) indicates similar kinetics.

We investigated the high temperature slow growth found for *S. cerevisiae tel1* and *tel2* mutants after 100–150 generations of subculturing in several of the *Sz. pombe* mutant strains in this study. Indeed we did find such a phenotype for some of the mutants, but it did not correlate with telomere length. Thus, *rad3* and *rad26* mutants (which have short telomeres) display slow growth at 37°C, whereas *rad1* and *rad17* strains (likewise with short telomeres) do not. There were also examples of mutants with normal telomeres; *hus1*, *rad21* and *wee1*, that did have the ts phenotype. In *Sz.*

pombe, then, this weak ts phenotype must be the consequence of some other property of checkpoint mutants (and other mutants) than their shortened telomeres. This also raises the possibility that the weak ts phenotype seen for the two *S. cerevisiae* mutants may not be a consequence of, but only coincide in time with, telomere shortening. Clearly, this temperature sensitivity is not a general property of *Sz. pombe* mutants with short telomeres.

RCK1 and *RCK2* of *S. cerevisiae* encode protein kinases of similar sequence (Dahlkvist and Sunnerhagen, 1994) and with similar effects in *Sz. pombe* checkpoint mutants, namely, to increase radiation and hydroxyurea resistance (Dahlkvist *et al*., 1995). We originally interpreted this effect to be rather indirect, and to result solely from an imposed lengthening of the G_2 phase of the cell cycle in these mutants, thus compensating for the lack of G_2 delay upon DNA damage or perturbation of DNA replication. However, our present finding that expression of at least *RCK1* at high levels in *rad1* mutant cells will attenuate telomere shortening indicates that the functional relationship between these two protein kinases and checkpoint proteins may be closer than previously suspected. None of the mutations interfering with the G_2-to-M transition studied in this work (*cdc2-1w*, *cdc2-3w*, Δ*wee1*, *adh:cdc25*) affected telomere length, arguing against the possibility that simply spending a longer time in G_2 would make telomeres grow longer. High levels of *RCK1* expression were able to increase telomere length even in a wild-type background. This can be interpreted as *RCK1* acting downstream in the signalling pathway from the checkpoint proteins, analogous to our previous finding that *RCK1* expression will cause cell elongation even in a wild-type background (Dahlkvist *et al*., 1995). The authentic roles of *RCK1* and *RCK2* in *S. cerevisiae* remain obscure, and in this context it will be relevant to investigate whether overexpression or disruption of these genes will affect telomere size in *S. cerevisiae*.

Although a distinct shortening of telomeres was caused by disruption of the *rad1*$^+$ gene, only a marginal effect on loss rate of linear or circular minichromosomes was seen. The finding that the increase in loss rate was higher with the linear minichromosome (2-fold) than with the circular (1.5-fold) might be taken as evidence that it is the result of a telomere defect given that circular chromosomes lack telomeres. Indeed, for the *S. cerevisiae* Δ*tel1* mutation, a similar modest increase in linear chromosome loss rate (~3-fold) was found (Greenwell *et al*., 1995). However, we think such a conclusion is not warranted, given the marginal difference between the two situations and the small overall increase. Increased chromosome loss rates above the level of *rad1* mutants have previously been observed in mutant *Sz. pombe* strains shown here not to have shortened telomeres, e.g. *chk1* (Griffiths *et al*., 1995), *rad8*, or *rad13* (Murray *et al*., 1994). Clearly factors other than length of telomeres determine telomere function as assayed by chromosome stability. Apparently, it is possible for a cell to have drastically shortened telomeres without a profound effect on telomere function as seen in this type of assay.

Defects in DNA metabolism near telomeres in *S. cerevisiae*, such as in *cdc13* mutants, activate the *RAD9*-dependent checkpoint (Weinert and Hartwell, 1993; Weinert *et al*., 1994; Garvik *et al*., 1995). Elimination of an entire telomere activates the *RAD9*-dependent checkpoint (Sandell and Zakian, 1993); however, inactivation of telomerase does not (Garvik *et al*., 1995; Lin and Zakian, 1995). A straightforward model of the situation in *Sz. pombe* would be that lack of activation of the replication checkpoint on shortening of telomeres allows them to decrease from wild-type length to the equilibrium length seen in checkpoint mutants after >100 generations of subculturing. Then, presumably another mechanism for maintenance of telomere size sets in, inasmuch as telomeric repeats do not disappear completely. For the *rad1*$^+$ gene, a case could be made for a more direct involvement in DNA metabolism at telomeres. The Rad1 product has sequence similarity to the *Ustilago maydis* Rec1 protein (Long *et al*., 1994), for which a 3′→5′ exonuclease activity has been found in vitro (Thelen *et al*., 1994). The *S. cerevisiae* Rad17 protein is also similar to these two proteins (Lydall and Weinert, 1995; Siede *et al*., 1996). Garvik *et al.* (1995) found that *cdc13* mutants accumulate single-stranded DNA near telomeres, corresponding to the T/G-rich strand. Based on these observations a model has been proposed (Lydall and Weinert, 1995) where Rad17 degrades one strand of DNA near sites of DNA damage, such as in the vicinity of telomeres in *cdc13* mutants. By analogy, one could envisage *Sz. pombe* Rad1 performing some step essential for, e.g., processing of telomeres before elongation by, e.g., telomerase. These two models are of course in principle compatible with each other. DNA polymerases have been demonstrated to be part of the replication checkpoint (Durso *et al*., 1995; Francesconi *et al*., 1995; Navas *et al*., 1995), and it is reasonable to expect a DNA repair/DNA-processing enzyme to be part of a DNA damage checkpoint. However, the situation in *Sz. pombe* is not quite the same as the one in *S. cerevisiae*, since *rad1*$^+$ is required for both the replication and the G_2 DNA damage checkpoints, whereas *RAD17* is not required for the replication checkpoint (Weinert *et al*., 1994). Also, the state discussed by Lydall and Weinert (1995) and Garvik *et al.* (1995) is not analogous to that studied here, because we demonstrate telomere shortening directly in checkpoint mutants, whereas their work deals with checkpoint activation after mutation in the telomere-binding protein, Cdc13. Further, a more general model must accommodate the fact that not only *rad1* mutants but four of the replication checkpoint-

deficient mutants have shortened telomeres. We therefore think it is possible that, apart from their general roles in maintaining genomic integrity, the Rad1, Rad3, Rad17, and Rad26 proteins participate together in signalling different aberrations from the normal state of telomeres, be it short telomeric repeats or, e.g., single-stranded regions. To find out about these mechanisms, it will be elucidating to see whether these proteins can be found physically associated with telomeres in vivo. Equally relevant is the issue of whether the clustering of telomeres in G_2 seen in wild-type *Sz. pombe* (Funabiki *et al*., 1993) is affected in *rad1*, *rad3*, *rad17*, and *rad26* mutants, which have shortened telomeres.

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