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Effects of Genome Position and the DNA Damage Checkpoint on the Structure and Frequency of *sod2* Gene Amplification in Fission Yeast

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The *Schizosaccharomyces pombe sod2* gene, located near the telomere on the long arm of chromosome I, encodes a Na^+ (or Li^+)/H^+ antiporter. Amplification of *sod2* has previously been shown to confer resistance to LiCl. We analyzed 20 independent LiCl-resistant strains and found that the only observed mechanism of resistance is amplification of *sod2*. The amplicons are linear, extrachromosomal elements either 225 or 180 kb long, containing both *sod2* and telomere sequences. To determine whether proximity to a telomere is necessary for *sod2* amplification, a strain was constructed in which the gene was moved to the middle of the same chromosomal arm. Selection of LiCl-resistant strains in this genetic background also yielded amplifications of *sod2*, but in this case the amplified DNA was exclusively chromosomal. Thus, proximity to a telomere is not a prerequisite for gene amplification in *S. pombe* but does affect the mechanism. Relative to wild-type cells, mutants with defects in the DNA damage aspect of the rad checkpoint control pathway had an increased frequency of *sod2* amplification, whereas mutants defective in the S-phase completion checkpoint did not. Two models for generating the amplified DNA are presented.

INTRODUCTION

Elaborate mechanisms have evolved to ensure the faithful replication and segregation of genetic material. Gene amplification, a relative increase in the copy number of a fraction of the genome, is a useful assay for genomic instability and has broad consequences for the organism in which it occurs. Amplification generates redundant copies of genes, which are less subject to mutational pressures and therefore can serve as raw material for the evolution of new functions (Britten and Davidson, 1971). Amplification of oncogenes is an important aspect of tumorigenesis (Alitalo and Schwab, 1986), whereas amplification of genes that encode the pharmacological targets of chemotherapeutic drugs leads to drug resistance (Giai *et al.*, 1991). In some cases, the amplification of specific genes is regulated developmentally. During oogenesis in *Drosophila melanogaster* the chorion genes become amplified through repeated initiations of DNA replication in a specific region, forming an onion skin structure (Orr-Weaver *et al.*, 1989). Amplification of the rRNA genes in *Xenopus laevis* proceeds by a rolling circle mechanism (Brown and Dawid, 1968). In mammalian cells, developmentally programmed amplifications are not known to occur, but numerous studies have explored the spontaneous gene amplifications that arise stochastically and confer resistance to specific toxic agents. Investigations of the very early events of gene amplification by fluorescence in situ hybridization have demonstrated the importance of chromosome–chromosome fusion events in generat-

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ing dicentric chromosomes, which subsequently participate in bridge–breakage–fusion cycles (Smith *et al.*, 1990; Toledo *et al.*, 1992). These observations have led to models in which the initiating event of gene amplification is either loss of telomeric sequences (Smith *et al.*, 1995) or breakage of chromosomes (Windle *et al.*, 1991).

Primary mammalian cells are not permissive for amplification, with a spontaneous rate of $<10^{-9}$ per cell per generation, whereas immortalized cell lines amplify genes at rates of $\sim10^{-4}$–10^{-5} per cell per generation (Tlsty *et al.*, 1989; Wright *et al.*, 1990). Permissivity for amplification is recessive (Tlsty *et al.*, 1992), and the tumor suppressor gene p53 has been shown to be an important participant, because cells with an inactive p53 pathway are permissive for amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Furthermore, variants of permissive cell lines can be isolated that have an increased amplification rate (Giulotto *et al.*, 1987). As important as these and other studies of mammalian cells have been in addressing the mechanisms and regulation of gene amplification, they lack the ability to use genetics readily to aid in identifying the proteins involved.

The budding yeast *Saccharomyces cerevisiae* has been used to study gene amplification. Characterization of strains selected for overexpression of *ACP1, CUP1, ADH2, ADH4, DFR1,* and *URA2* has revealed several classes of amplified DNA (amplicons), including direct and inverted repeats, which may be chromosomal or extrachromosomal. *CUP1* amplicons are chromosomal, tandem direct repeats (Fogel and Welch, 1982) but represent a special case, because the parental strain already carries a tandem duplication of the gene, and a primary amplification event, starting from one copy, has never been observed experimentally. Gene amplifications accompanied by translocations have been reported for the *ACP1* (Hansche *et al.*, 1978), *URA2* (Bach *et al.*, 1995), and *ADH2* genes (Paquin *et al.*, 1992), and several different amplicon structures have been reported. *ADH2* amplicons are chromosomal, dispersed, direct repeats that became translocated to rDNA, whereas *DFR1* amplicons are circular and extrachromosomal, with two copies of the *DFR1* gene in an inverted orientation (Huang and Campbell, 1995). *ADH4* amplicons can be found either as chromosomal duplications or as extrachromosomal, linear elements in an inverted orientation (Dorsey *et al.*, 1992).

The fission yeast *Schizosaccharomyces pombe*, another excellent model system for investigating gene amplification, has notable differences in chromosome organization from *S. cerevisiae*, which might be reflected in differences in the mechanisms of gene amplification. Both organisms have approximately the same amount of DNA (14 Mb), but whereas *S. cerevisiae* has 16 chromosomes, ranging in size from 225 kb to 2.2 Mb, the three *S. pombe* chromosomes range in size from 3.5 to 5.7 Mb, and *S. pombe* centromeres more closely resemble those of mammalian cells (Ngan and Clarke, 1997). Compared with *S. cerevisiae*, the integration of transforming DNA by nonhomologous recombination is much more frequent in *S. pombe* and mammalian cells, suggesting that the processing of this DNA in these organisms may be similar. Furthermore, as with *S. cerevisiae*, many *S. pombe* mutants are available that have defects in various aspects of cell cycle checkpoint control and in DNA repair, allowing the effects of these mutations on gene amplification to be studied.

Two examples of gene amplification in *S. pombe* are known. First, duplication of three temperature-sensitive alleles of *cdc2,* through unequal crossing over between flanking 5S RNA genes, is responsible for their high reversion frequency (Carr *et al.*, 1989). Second, the nitrosoguanidine-induced, chromosomal amplification of *sod2*, which encodes a Na^+ (or Li^+)/H^+ antiporter located in the plasma membrane (Dibrov *et al.*, 1997), conferred Li resistance, although the mechanism of amplification was not characterized (Jia *et al.*, 1992). We have now isolated several independent strains carrying spontaneous amplifications of *sod2* and have analyzed the structure of the amplified DNA. We have mapped the *sod2* locus to a telomere-proximal position on chromosome I. By moving *sod2* to a telomere-distal locus, we show that proximity to a telomere is not necessary for amplification but does influence amplicon structure. We also show that mutants defective in the DNA damage checkpoint have a greatly increased frequency of *sod2* amplification.

MATERIALS AND METHODS

Cell Strains and Growth

The strains used were *h*$^-$, *ade6-M210; h*$^-$, *ade*$^-$, *leu*$^-$, *ura4-D18; h*$^-$, *rad1-1*; *h*$^-$, *rad3.136, ura4-D18*; *h*$^-$, *rad9.192, ade6-704, ura4-D18* (Al-Khodairy and Carr, 1992); *h*$^-$, *leu1.32; rad17.d*; *h*$^-$, *rad17.F, ura4-D18, leu1.32, ade6-704*; *h*$^-$, *rad26.d, ura4-D18, leu1.32, ade6-704*; *h*$^-$, *rad27.d, ura4-D18, leu1.32, ade6-704*; and *h*$^-$, *rad26.T12, ura4-D18, leu1.32, ade6-704* (Al-Khodairy *et al.*, 1994). Media (YE5S and Edinburgh minimal medium) were prepared, and cell culture was carried out as described previously (Moreno *et al.*, 1991; Jia *et al.*, 1992). The *sod2*-specific probe used was the 2.4-kb *Hin*dIII fragment from pSOD2.4 (Jia *et al.*, 1992). The *S. pombe* telomere probe used was the 0.8-kb *Taq*I fragment from pSPT-16 (Sugawara, 1989).

Physical Mapping of sod2 and gln1

sod2 was mapped onto the *S. pombe* genome by using the ordered set of cosmid and P1 clones available from the Genome Analysis Laboratory (RessourcenZentrumPrimärDatenbank, Berlin, Germany). The 2.3-kb *Hin*dIII fragment (Hoheisel *et al.*, 1993) was radiolabeled and hybridized to the filters. Because this cosmid library is incomplete near the telomere of chromosome I, which contains *Not*I fragment L, the location of *sod2* was confirmed by analyzing Southern transfers of cosmid DNA from another ordered cosmid library (Mizukami *et al.*, 1993).

Reciprocal Exchange of sod2 and gln1

The plasmid psod2::ura was constructed by blunting the ends of the 1.8-kb *Hin*dIII fragment of the *ura4* gene by using the Klenow fragment of DNA polymerase and inserting the fragment into the blunt-ended *Bst*EII site of pSOD2.4 (Jia *et al.*, 1992). The plasmid pgln1::LEU2 was constructed by blunt-end ligating the 2.2-kb *Hin*dIII fragment of *LEU2* into the blunt-ended *Bst*EII site of pGLN1 (Barel *et al.*, 1988). The plasmid pgln1::sod2 was constructed by blunt-end ligating the 2.4-kb sod2 *Hin*dIII fragment of pSOD2.4 into the blunt-ended *Bst*EII site of pGLN1. The plasmid psod2::gln1 was constructed by blunt-end ligating the 3.2 kb *gln1 Hin*dIII fragment of pGLN1 into the blunt-ended *Bst*EII site of pSOD2.4. The 4.2-kb *Hin*dIII fragment of plasmid psod2::ura4 and the 5.4-kb *Hin*dIII fragment of plasmid pgln1::LEU2 were transformed independently into a strain of genotype h^-, *ura4-D18, leu1-32*, and ura$^+$ or leu$^+$ transformants were selected, respectively, to obtain *sod2::ura4*$^+$ and *gln1::LEU2* strains. *sod2::ura4*$^+$ cells are highly sensitive to Li, and *gln1::LEU2* cells are glutamine auxotrophs. The *sod2::ura4*$^+$ strain was transformed with the 5.6-kb *Hin*dIII fragment of plasmid pgln1::sod2 and transformants selected for increased resistance to LiCl were screened for glutamine auxotrophy to obtain a *sod2::ura4*$^+$, *gln1::sod2*$^+$ strain. Similarly, the *gln1::LEU2* strain was transformed with the 5.6-kb *Hin*dIII fragment of plasmid psod2::gln1, and glutamine prototrophs were selected, thereby obtaining a strain that is *gln1::LEU2*, *sod2::gln1*$^+$. This strain was backcrossed *to* h^+, *ura4-D18, leu1-32* cells to obtain h$^+$, gln1$^-$::LEU2$^+$, sod2$^-$::gln1$^+$. This strain was crossed with the h^-, *sod2::ura4*$^+$, *gln1::sod2*$^+$ strain, and ura$^+$, leu$^+$ haploid prototrophs were selected to produce the h^-, *sod2::gln1+; gln1::sod2+, ura4-D18, leu1-32* strain TPSXG.

Selection of Strains Carrying Amplifications of sod2

Cells were grown in YE5S medium at 30°C in a shaking water bath to midlog phase, harvested by centrifugation at 1800 × *g*, washed twice with water, and resuspended in water at 1×10^9 cells/ml. Aliquots of 5×10^7 cells were plated directly onto selective plates (Edinburgh minimal medium plus appropriate supplements, 40 mM LiCl, pH 5.0) and incubated at 30°C. Colonies were scored 4–5 d after plating. Strain TPSXG, in which the *sod2* and *gln1* genes were reciprocally exchanged, is ~2.5 times more sensitive to LiCl than parental cells (our unpublished results). Therefore, LiCl-resistant variants of TPSXG were selected in 16 mM LiCl. The LiCl sensitivity of all of the rad mutant strains to LiCl was the same as that of wild-type cells.

Copy Number Determination

Gene copy number was determined either by quantitative Southern analysis or by a slot blot technique (Patterson *et al.*, 1995).

Contour-clamped Homogeneous Electric Field (CHEF) Gel Electrophoresis

Agarose plugs containing *S. pombe* genomic DNA or *Not*I digestion products were prepared as previously described (Alfa *et al.*, 1993). Electrophoresis was performed using a Chef Mapper XA system (Bio-Rad, Hercules, CA). The electrophoresis conditions were 1% chromosomal grade agarose, 0.5× Tris borate-EDTA, 6 V/cm, 14°C, and 120° included angle. The pulse time was 60 s for the first 15 h and 90 s for the following 9 h. When indicated, ethidium bromide was included at 0.05 μg/ml in the gel and buffer. Exonuclease III digestions and topoisomerase I treatments of chromosomal DNA in agarose plugs were conducted as previously described (Beverley, 1988).

Table 1. Distribution of *sod2* copy number in independent Li-resistant strains

Strain	No. of colonies without amplification	No. of colonies with amplification	Mean ± SD of copy number
1	0	6	4.8 ± 0.7
2	2	5	3.3 ± 1.0
3	3	5	3.7 ± 2.0
4	2	5	3.6 ± 1.6
5	1	7	4.2 ± 1.3
6	1	7	4.7 ± 1.2
7	3	3	2.0 ± 1.7
8	3	4	2.6 ± 1.5
9	2	5	3.2 ± 1.4
10	1	6	3.9 ± 1.3
11	1	7	3.6 ± 1.1
12	2	5	4.3 ± 2.1
13	1	6	4.1 ± 1.4
14	0	7	3.6 ± 0.9
15	2	6	3.6 ± 1.8
16	1	7	3.8 ± 1.0
17	1	7	4.0 ± 1.0
18	1	6	3.6 ± 1.2
19	0	8	4.4 ± 0.5
20	3	5	2.7 ± 1.5
wt	7	0	1.0 ± 0.05

RESULTS

sod2 Is Located on the Long Arm of Chromosome I, Near the Telomere

To aid in the structural analysis of *sod2* amplicons, we mapped the *sod2* locus by using ordered cosmid and P1 library filters. *sod2* is on the long arm of chromosome I, on the telomeric *Not*I fragment L and *Sfi*I fragment H (Fan *et al.*, 1991), between *rad8* and the telomere. This assignment was confirmed by demonstrating genetic linkage between *sod2* and *rad8*. Based on the hybridization pattern of a *sod2* probe with a minimally overlapping set of cosmids from this region (Mizukami *et al.*, 1993), we estimate that *sod2* is ~35–90 kb from the telomere (our unpublished results).

sod2 Amplicons Are Extrachromosomal

To investigate the mechanisms of gene amplification, 20 independent populations were grown from single cells, and LiCl-resistant variants were selected from each. The *sod2* copy number was determined for several colonies from each population (Table 1). Every LiCl-resistant population showed evidence of *sod2* amplification, but within some populations not every colony contained a detectable increase in *sod2* copy number.

To investigate the amplified DNA, chromosomal DNA from a single amplified strain from each population was digested with *Not*I, separated by CHEF gel electrophoresis, and analyzed with a *sod2* probe. Con-

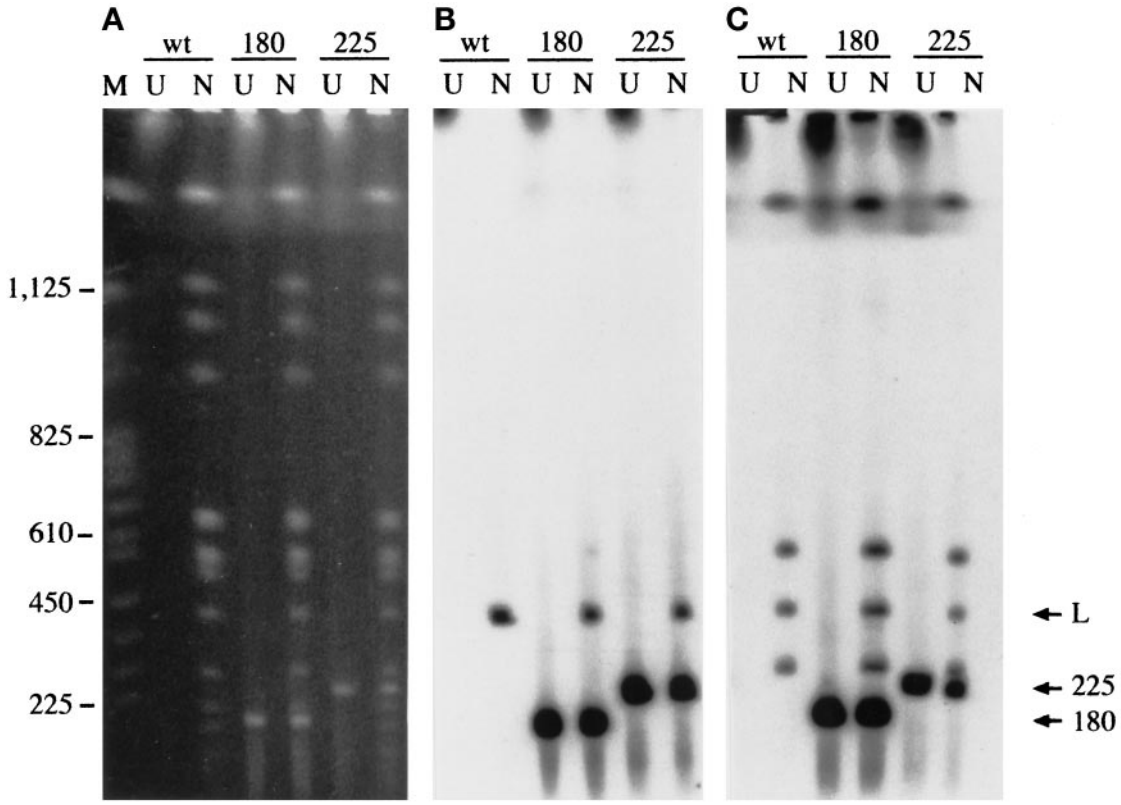

Figure 1. Pulsed field gel analysis of amplified *sod2* DNA. Undigested (U) or *Not*I-digested (N) total chromosomal DNA samples from wild-type and two representative amplified strains were separated by CHEF gel electrophoresis. (A) Ethidium bromide–stained gel. (B) Southern transfer probed with a *sod2* probe. (C) Southern transfer probed with telomere probe. The lanes marked 225 and 180 contain DNA from a strain carrying the 225- or 180-kb amplicon, respectively; wt, wild-type; L, *Not*I restriction fragment L, which contains the endogenous *sod2* gene; 225 and 180, amplified *sod2* DNA. *S. cerevisiae* chromosomes in agarose plugs (Life Technologies, Bethesda, MD) were used as *DNA* size markers (left) and are given in kilobases.

sistent with our mapping results, in wild-type cells the probe hybridized to *Not*I fragment L. Two hybridization patterns were observed in the 20 strains containing *sod2* amplicons (representative examples in Figure 1). In 16 strains, *sod2* sequences were detected not only in *Not*I fragment L but also in an extra band that comigrated with the 225-kb *S. cerevisiae* chromosome I. In four strains, *sod2* sequences were detected in *Not*I fragment L and in an extra band with an apparent size of 180 kb. Estimation of the amount of *sod2* signal in the amplicon bands, using the *Not*I fragment L as an internal control, indicated that there are approximately seven to nine extra copies of *sod2* in strains carrying amplicons. This should be considered a lower limit, because we cannot exclude the possibility that amplification of sod2 has occurred within fragment L as well. We think this unlikely, given the fact that the size of fragment L is unchanged. Because the migration of *sod2* amplicons is unaffected by *Not*I digestion (Figure 1, A and B), we conclude that they are extrachromosomal. Furthermore, within the resolution of the CHEF gels, all the other chromosomal *Not*I digestion products from the mutant strains comigrate with the corresponding wild-type fragments, suggesting that gross rearrangements do not occur during formation of the amplicons. Because *sod2* maps near a telomere of chromosome I, we determined whether the amplicons also contained telomeric sequences. A telomere probe (Sugawara, 1989) hybridizes to the 225 and 180 kb bands and to the *Not*I bands known to contain telomeres (Figure 1C).

The sod2 Amplicons Are Linear

Because the amplicons migrate in CHEF gels as tight bands, they are either linear molecules of the apparent size or much smaller, covalently closed, supercoiled circles (Beverley, 1988). To determine the topology of the amplicons, we treated total genomic DNA in agarose plugs with exonuclease III, which degrades linear but not circular DNA, or with topoisomerase I, which relaxes supercoiled circular DNA and thereby reduces its mobility during electrophoresis. As expected for linear molecules, the 225-kb band is degraded by exonuclease III, as are the linear *S. cerevisiae* chromosomes used as markers (Figure 2A). Furthermore, mi-

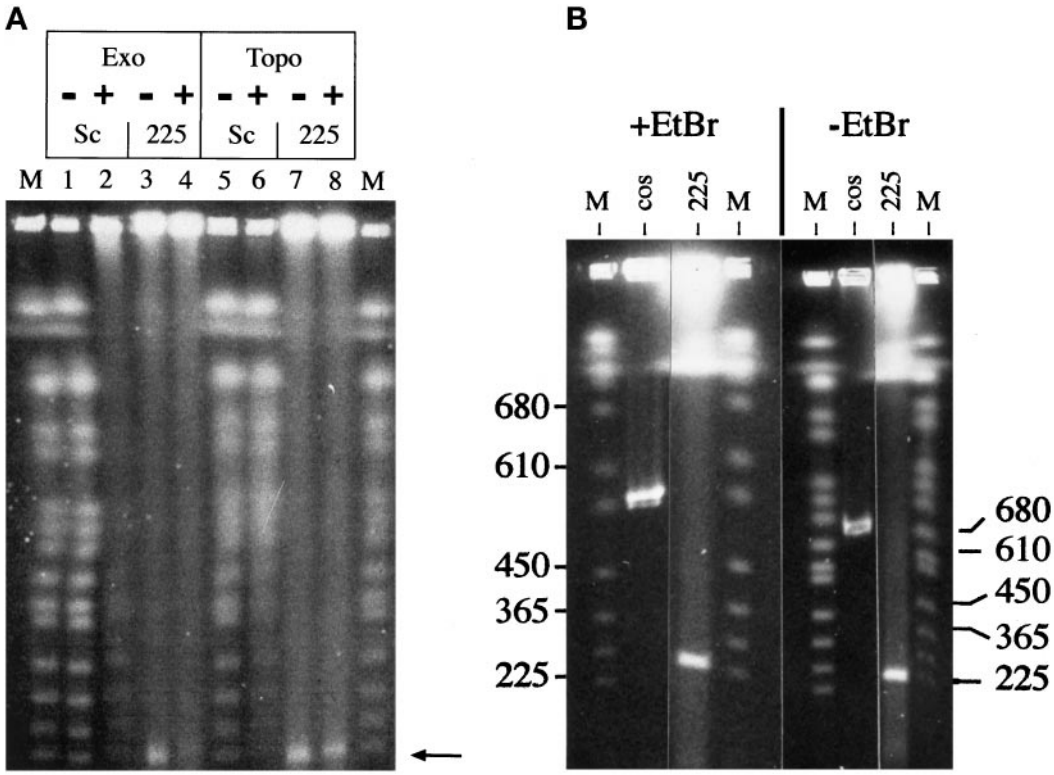

Figure 2. Topology of amplified *sod2* DNA. (A) Total chromosomal DNAs from *S. cerevisiae* (Sc) and an *S. pombe* LiCl-resistant strain with the 225-kb amplicon (225) were treated (+) or mock-treated (−) with either exonuclease III (lanes 1–4) or topoisomerase I (lanes 5–8) and analyzed by CHEF electrophoresis. (B) Effect of ethidium bromide on electrophoretic mobility. Total chromosomal DNAs of *S. cerevisiae* (Sc), an *S. pombe* LiCl-resistant strain with the 225-kb amplicon (225), and a cosmid (cos) were separated on CHEF gels in the presence or absence of ethidium bromide (0.05 μg/ml). DNA size markers (left) are in kilobases.

gration of the 225-kb band is unaffected by topoisomerase I, indicating that it is not a supercoiled circular molecule. Similar results were obtained with the 180-kb amplicon (our unpublished results).

To confirm the linear structure of the amplicons, we took advantage of the differential migration of circular and linear DNAs in the presence of low concentrations of ethidium bromide (Beverley, 1988). Without ethidium bromide, a circular cosmid of ~35 kb migrates with an apparent size of ~700 kb, whereas in the presence of 0.05 μg/ml ethidium bromide, the same cosmid migrates with an apparent size of ~570 kb (Figure 2B). In contrast, the migration of the 225-kb band was unaffected. Similar results were obtained with the 180-kb amplicon (our unpublished results).

Is Proximity to a Telomere Necessary for sod2 Amplification?

To address this question, we constructed TPSXG, a strain in which *sod2* was reciprocally exchanged with *gln1*, which is located on the same arm of chromosome I as *sod2* but more than 1.5 Mb from the telomere. *gln1* is on *Not*I fragment D and also on *Sfi*I fragment E, between *rad4* and *ercc3sp* (Hoheisel *et al.*, 1993). LiCl-resistant variants were selected from 20 independent populations of TPSXG, and CHEF gel electrophoresis of chromosomal DNA was performed on uncut and *Not*I-digested samples. Four different patterns of *sod2* hybridization to *Not*I-digested DNA were observed in the 20 strains containing *sod2* amplicons (representative examples in Figure 3). In the parental strain *sod2* sequences were detected in *Not*I fragments D and L, as expected. These wild-type fragments were present in all of the LiCl-resistant strains, in addition to new fragments containing *sod2*. In 12 Li-resistant strains, *sod2* sequences were also detected in an extra band of ~780 kb (Figure 3B). In six strains, *sod2* sequences were also detected in an extra band of ~1400 kb (Figure 3B). In one strain, *sod2* sequences were also detected in an extra band of ~960 kb (Figure 3B), and in another strain, *sod2* sequences were also detected in two extra bands of ~960 and ~570 kb (Figure 3B). Because the *sod2* amplicons migrate with chromosomal DNA in samples untreated with *Not*I (Figure 3A), we conclude that they are chromosomal. Considering the large sizes of the amplicon bands, it is some-

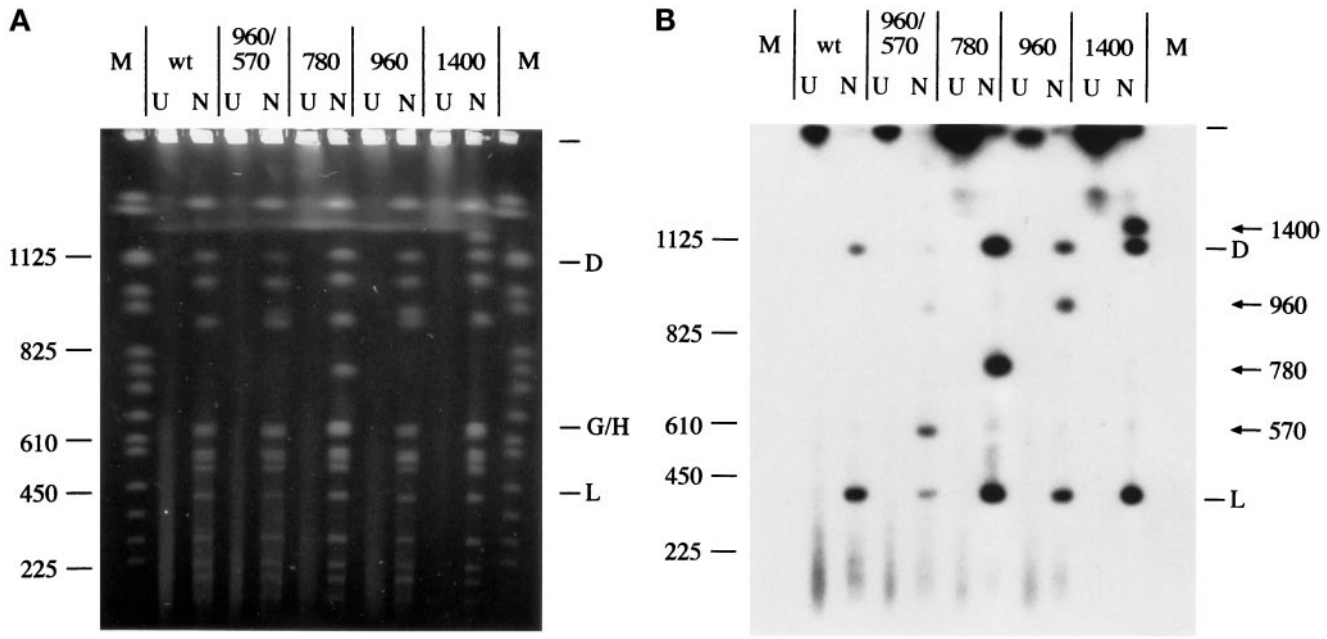

Figure 3. Pulsed field gel analysis of amplified DNA in strains with *sod2* at the *gln1* locus. Undigested (U) or *Not*I-digested (N) total chromosomal DNA samples from wild-type (wt) and four representative *sod2*-amplified strains were separated by CHEF gel electrophoresis. Lanes are marked with the corresponding size in kilobases of the extra *sod2*-containing fragments. (A) Ethidium bromide–stained gel. (B) Southern transfer probed with a *sod2* probe. *Not*I restriction fragments D (contains *gln1*), G/H, and L (contains *sod2*) are labeled. Arrows denote the amplified *sod2* DNA. *sod2* hybridization to *Not*I fragment L in these strains is due to residual *sod2* sequences at the normal locus. *S. cerevisiae* chromosomes in agarose plugs (Life Technologies) were used as *DNA* size markers (left) and are given in kilobases.

what surprising that, within the resolution of the CHEF gels, only one strain exhibited a difference in the migration of the other chromosomal *Not*I digestion products. In strain 960, the doublet containing *Not*I fragments G and H, normally observed in a wild-type strain, is now a single band (Figure 3A, lane 960). Because *Not*I fragment H is adjacent to D, the new locus of *sod2*, it is likely that the rearrangement that gave rise to the 960-kb fragment involved sequences from fragment H.

Cell Cycle Checkpoint Control of sod2 Gene Amplification

Normal human cells are not permissive for gene amplification but become so when pathways that monitor DNA damage are compromised (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Therefore, mutant strains of *S. pombe* defective in cell cycle checkpoint controls might also have an increased frequency of gene amplification. To address this issue, we assayed several checkpoint control mutants for the frequency of *sod2* amplification and for the structures of the amplified DNA. Strains with null mutations in *rad1, rad3, rad9, rad17,* and *rad26* all had an increased frequency of LiCl resistance compared with wild-type strains (Figure 4), and *sod2* was amplified in all LiCl-resistant strains (our unpublished results). These mutants are defective in both a DNA damage checkpoint that arrests the cell cycle after UV irradiation (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992) and in an S-phase completion checkpoint that delays the entry into mitosis of hydroxyurea-treated cells (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). To discriminate between these two checkpoints, we assayed mutants in which only one is defective. A rad27-null strain (*rad27* is also known as *chk1*; Walworth *et al.*, 1993), defective in the DNA damage checkpoint but with an intact S-phase completion checkpoint (Al-Khodairy *et al.*, 1994), also shows an increased frequency of *sod2* amplification compared with wild-type cells (Figure 4). These LiCl-resistant strains harbor 225- or 180-kb extrachromosomal amplicons, as do LiCl-resistant wild-type cells (our unpublished results). Conversely, strains with either the *rad17-F* or *rad26-T12* allele, with a defective S-phase completion checkpoint but an intact DNA damage checkpoint, show no increase in the frequency of *sod2* amplification relative to wild-type cells (Figure 4).

DISCUSSION

Amplification of *sod2* was previously shown to occur after mutagenic treatment with nitrosoguanidine, or upon stepwise selection with LiCl, and was sufficient

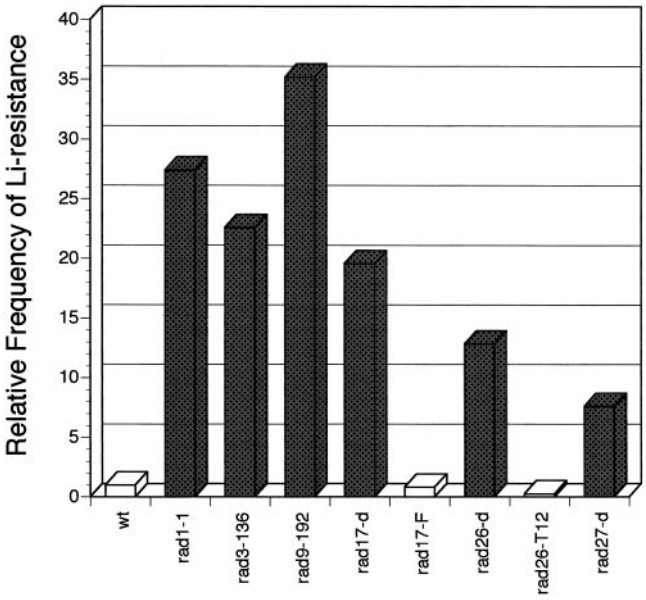

Figure 4. Frequencies of LiCl resistance in rad checkpoint control mutants. The indicated mutant strains and a wild-type strain were selected with LiCl. The frequencies have been normalized to that of the wild-type strain. Dark bars represent strains with a defective DNA damage checkpoint pathway. Light bars represent strains with an intact DNA damage checkpoint pathway.

to confer resistance to 40 mM LiCl (Jia *et al.*, 1992). The only observed mechanism of resistance in spontaneous LiCl-resistant strains is amplification of *sod2*, with 20 of 20 independent LiCl-resistant strains containing amplifications. Within each population, the *sod2* copy number was estimated to be from one to eight, with an average between four and five. Colonies lacking detectable amplification, within a population in which the gene is clearly amplified, could result from phenotypic lag, in which the amplified DNA is lost but the cells remain resistant to LiCl for some time, because the Sod2 protein still resides in the plasma membrane. Consistent with this hypothesis, the LiCl-resistant phenotype is gradually lost from populations in the absence of selection (Jia *et al.*, 1992; our unpublished results).

Analysis of the genomic DNA of all 20 independent populations in CHEF gels revealed that the *Not*I digestion pattern differed from that of wild-type cells by the appearance of a new band that migrated with an apparent size of either 225 kb (16 of 20 strains) or 180 kb (4 of 20 strains). These new bands hybridized to a *sod2* probe, indicating that they were responsible for the resistant phenotype, and to a telomere probe. Because the 225- and 180-kb bands were degraded by exonuclease III, were unaffected by topoisomerase I, and were as sensitive as linear controls to the presence of ethidium bromide in CHEF gels, they are linear. We hypothesize that telomeres are present on both ends of these linear amplicons, because their apparent sizes are stable during long-term growth under selective conditions (our unpublished results). The sizes of the chromosomal *Not*I fragments in LiCl-resistant and wild-type cells appear normal, suggesting that gross rearrangements did not occur when the amplified DNA was formed.

We physically mapped *sod2* to the long arm of chromosome I, 35–90 kb from the telomere. Previously, *sod2* had been mapped genetically to chromosome II, using LiCl resistance as a genetic marker in a strain that contained amplified *sod2* (Jia *et al.*, 1992). In the earlier work, a translocation of *sod2* from chromosome I to chromosome II probably occurred during nitrosoguanidine-induced amplification.

To determine whether the proximity of *sod2* to a telomere is necessary for its amplification, we constructed a strain in which *sod2* was reciprocally exchanged with *gln1*, which is in the middle of the same arm of chromosome I. LiCl-resistant mutants derived from this strain contain extra copies of *sod2*, but, in contrast to the extrachromosomal *sod2* amplicons from the wild-type strain, the extra copies are on relatively large, chromosomal *Not*I fragments. These results indicate that the amplification of *sod2* is not dependent on its proximity to a telomere, but the different amplicon structures observed in the two backgrounds suggest that different mechanisms are likely to be involved. Surprisingly, in the absence of selection there is little difference in the rate of loss of chromosomal amplicons in the swapped strain compared with extrachromosomal amplicons in the wild-type strain (our unpublished results). Amplification is likely to occur through more than one mechanism, and structure and stability of the amplicon observed will be influenced by positive or negative selection for coamplified genes.

The ease of selecting strains of *S. pombe* carrying *sod2* amplicons has allowed us to investigate the effect of the DNA damage and S-phase completion checkpoints on gene amplification. By using mutants that discriminate between these two, we determined that a defective DNA damage checkpoint leads to a substantial increase in the frequency of *sod2* gene amplification and that a defective S-phase completion checkpoint does not. The structure of the amplified DNA in a *rad27/chk1* null strain is indistinguishable from that in a wild-type background, suggesting that, although the mechanism is not affected, the absence of a DNA damage checkpoint increases the probability of amplification. DNA structures that would ordinarily be detected by the DNA damage checkpoint and repaired during the ensuing cell cycle arrest might, in the absence of the checkpoint, be more likely to enter a processing pathway leading to gene amplification. Progress through the cell cycle in the presence of such a structure might lead to an increase in homologous

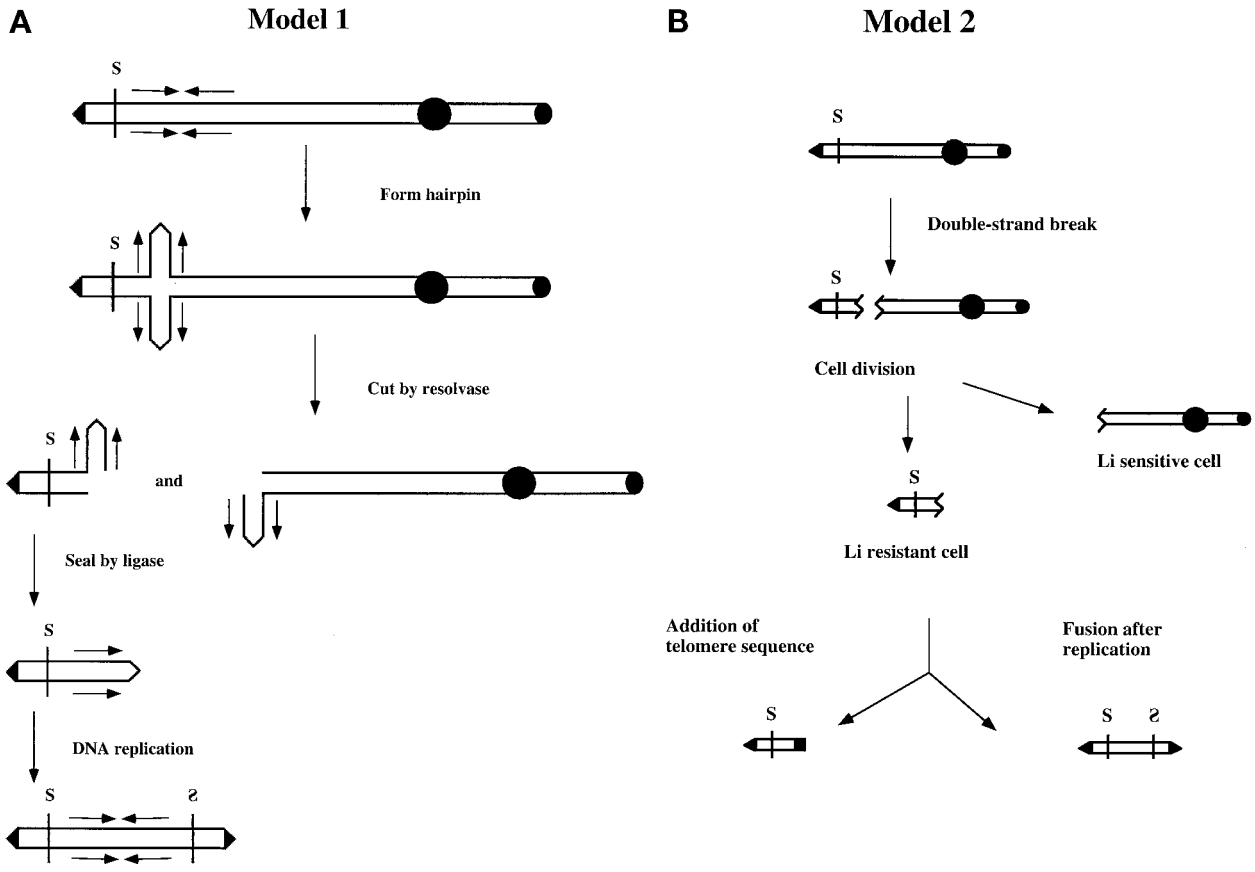

Figure 5. Two models for generating *sod2* extrachromosomal, linear amplicons. DNA replication is presumed to have occurred before either event; the second copy of chromosome I has been omitted for clarity. S, *sod2*; large filled circles, centromeres; triangles, telomere from the long arm of chromosome I; small filled circles, telomere from the short arm of chromosome I; filled square, de novo telomere; arrows, inverted DNA repeats.

recombination or to an increase in double-strand breaks that can be processed into a *sod2* amplicon.

The observation that defects in the DNA damage checkpoint pathway lead to an increase in the frequency of *sod2* gene amplification is reminiscent of the effect of defects in the mammalian p53 pathway. In both cases, progression through the cell cycle in the absence of a checkpoint pathway leads to an increase in the frequency of gene amplification, suggesting that the initiating events in gene amplification may be similar, i.e., DNA damage. Further genetic studies can use the *sod2* system to identify additional genes that, when mutant, increase the frequency of gene amplification. Candidates include genes associated with DNA repair, DNA replication, and regulation of the cell cycle.

How are the *sod2* amplicons generated? The different structures observed when *sod2* is at different genomic loci argue for more than one pathway. The chromosomal amplicons in the swapped strain could arise by unequal crossing over between repetitive sequences flanking the *gln1* locus (Hoheisel *et al.*, 1993; Mizukami *et al.*, 1993), as has been described for the duplication of certain *cdc2* mutant alleles by recombination between 5S RNA genes (Carr *et al.*, 1989). Such recombinations would produce the chromosomal amplicons we observe. The lack of detectable size differences in the other *Not*I fragments in three of the four classes of amplicon, although surprising, must be interpreted with caution, because small differences in these large fragments could easily go undetected. We propose two models to explain the two sizes of linear, extrachromosomal amplicons found in LiCl-resistant strains derived from wild-type cells that retain an apparently full-length chromosome I (Figure 5). Because our experiments were performed in haploid cells, the retention of chromosome I leads to the conclusion that the initiating event occurred after S-phase, when sister chromatids are present. The models are not mutually exclusive, and it is possible that the amplicons are formed in multiple steps, because many rounds of cell division have occurred before we can

analyze their structure. Both models are consistent with our experimental data and include testable hypotheses and predictions.

In Model 1 (Figure 5A), inverted repeats between *sod2* and the centromere could form a hairpin, which might be processed by a resolvase. This mechanism is similar to that involved in *Tetrahymena* rDNA amplification and has been shown to occur in *S. cerevisiae* (Butler *et al.*, 1996). The *sod2* fragment would be repaired by ligase, and the other centromeric fragment would eventually be lost. Replication of the *sod2*-containing fragment would yield a linear, extrachromosomal amplicon with two copies of *sod2* in a perfect inverted repeat. This model predicts that the amplicon would contain telomeres only from the long arm of chromosome I.

In Model 2 (Figure 5B), a double-strand break could occur between the centromere and *sod2*, stimulated, for example, by a stalled replication fork or a fragile site, both of which have been implicated in gene amplification in mammalian cells (Windle *et al.*, 1991; Coquelle *et al.*, 1997). The cell that receives both the normal chromosome I and the *sod2* fragment would survive selection. The fragment, with a telomere only at one end, could be healed either by de novo addition of a telomere or by fusion at the nontelomeric end after DNA replication. The resulting amplicon would have either one copy of *sod2* or two copies as an inverted repeat, respectively.

The observation of only two distinct sizes of *sod2* amplicons argues against a completely nonhomologous recombination mechanism. In each model, more than one repeat element or breakage site participates in generating the amplicon. It will be useful to analyze the sequences flanking *sod2* for repeats when the sequence of this region is available. Further analysis of the structure of the *sod2* amplicons will provide better insight into the mechanism of gene amplification in *S. pombe*.

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