

# Mechanisms of *sod2* Gene Amplification in *Schizosaccharomyces pombe*

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Gene amplification in eukaryotes plays an important role in drug resistance, tumorigenesis, and evolution. The *Schizosaccharomyces pombe* *sod2* gene provides a useful model system to analyze this process. *sod2* is near the telomere of chromosome I and encodes a plasma membrane  $\text{Na}^+(\text{Li}^+)/\text{H}^+$  antiporter. When *sod2* is amplified, *S. pombe* survives otherwise lethal concentrations of LiCl, and >90% of the amplified *sod2* genes are found in 180- and 225-kilobase (kb) linear amplicons. The sequence of the novel joint of the 180-kb amplicon indicates that it is formed by recombination between homologous regions near the telomeres of the long arm of chromosome I and the short arm of chromosome II. The 225-kb amplicon, isolated three times more frequently than the 180-kb amplicon, is a palindrome derived from a region near the telomere of chromosome I. The center of symmetry of this palindrome contains an inverted repeat consisting of two identical 134-base pair sequences separated by a 290-base pair spacer. LiCl-resistant mutants arise 200–600 times more frequently in strains deficient for topoisomerases or DNA ligase activity than in wild-type strains, but the mutant cells contain the same amplicons. These data suggest that amplicon formation may begin with DNA lesions such as breaks. In the case of the 225-kb amplicon, the breaks may lead to a hairpin structure, which is then replicated to form a double-stranded linear amplicon, or to a cruciform structure, which is then resolved to yield the same amplicon.

## INTRODUCTION

Genomic alterations, often deleterious, can also confer unique advantages to a cell. Gene amplification, defined as a relative increase in copy number of a part of the genome smaller than a chromosome, provides a means to increase the amounts of certain proteins without changing the DNA sequence near the relevant genes (Wu and Black, 1987). Amplification plays important roles in drug resistance (Stark *et al.*, 1989), adaptive mutability (alterations in the specificity or rate of mutation during stress), and divergent evolution. Amplification also provides a selective advantage to many tumors, which often contain amplified oncogenes (Bishop, 1987). Amplification of a gene provides additional targets for mutation (Andersson *et al.*, 1998) and thus can be the first step in the evolution of a new gene, because the extra copies are free to provide new functions (Kimura and Ota, 1974).

Two main classes of amplified structures (amplicons) have been characterized. Tandem head-to-tail arrays predominate in prokaryotes. In examples from both *Escherichia coli* (Whoriskey *et al.*, 1987) and phage T4 (Wu *et al.*, 1991),

short (<20 base pairs [bp]), imperfect, direct repeats flank the duplicated region, suggesting that homologous recombination is a major mechanism. In the eukaryote *Leishmania*, homologous recombination between longer (541 bp) direct repeats flanking the P-glycoprotein gene produces amplicons harboring tandem duplications. Palindromic head-to-head amplification, which is widespread in many eukaryotes, including *Leishmania*, *Tetrahymena*, yeast, and mammals, has been observed as both intrachromosomal and extrachromosomal (linear and circular) amplified structures (Ford *et al.*, 1985; Yao *et al.*, 1985; Walton *et al.*, 1986; Hightower *et al.*, 1988; Ouellette *et al.*, 1991).

Determining amplification mechanisms has been difficult in mammalian cells, in large part because of limitations in analyzing the novel joints formed. Novel joints are created when genomic sequences from distinct locations in the wild-type genome become juxtaposed. The work of several laboratories has made it clear that dicentric chromosomes are often present early in amplification events in mammalian cells and that they can be generated by either telomere–telomere fusions (Smith *et al.*, 1992) or double-strand breaks, perhaps at fragile sites (Windle *et al.*, 1991; Coquelle *et al.*, 1997). The precise location of these breakage events is re-

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quired to determine the mechanism responsible for forming the amplification structures.

The fission yeast *Schizosaccharomyces pombe* provides an excellent model system for studying gene amplification. Its simple genome comprises three chromosomes, ranging in size from 3.5 to 5.7 megabases. Fission yeast is permissive for gene amplification, and spontaneous amplification has been observed at a frequency of  $\sim 10^{-6}$  per cell per generation (Patterson *et al.*, 1999). The *sod2* gene of *S. pombe* encodes a  $\text{Na}^+/\text{H}^+$  antiporter that is essential for  $\text{Na}^+$  export (Jia *et al.*, 1992). Amplification of *sod2* is the major mechanism by which cells become resistant to high concentrations of LiCl. Preliminary characterization of the predominant amplicons in LiCl-resistant cells revealed that they are extrachromosomal and, strikingly, of only two sizes, 225 or 180 kilobases (kb). The amplicons are linear, contain telomeric sequences, and are stable only if LiCl selection is maintained (Jia *et al.*, 1992; Patterson *et al.*, 1999). Genetic studies have indicated that mutant strains defective in the DNA-damage checkpoint, but not in the S-phase-completion checkpoint, have an increased frequency of *sod2* amplification (Patterson *et al.*, 1999). We now present a detailed structural characterization of the two predominant *sod2* amplicons and show that mutations likely to produce DNA lesions also enhance amplicon formation. The new data suggest specific models for amplicon formation.

## MATERIALS AND METHODS

### Selection of Strains Resistant to LiCl

The three temperature-sensitive mutant strains were *h<sup>-</sup>*, *top2-191*, *leu1-32*; *h<sup>-</sup>*, *top1::swi3*, *top2-110*, *leu1-32*; the strain defective in ligase function was *h<sup>+</sup>*, *cdc17-K42*. All were obtained from the laboratory of Paul Nurse. Supplemented yeast extract medium and Edinburgh minimal medium were described previously (Moreno *et al.*, 1991; Jia *et al.*, 1992). Selections were performed as described previously (Patterson *et al.*, 1999). All the mutant strains but one had the same sensitivity to LiCl as the wild-type strain, but the ligase-deficient strain, *cdc17*, was more sensitive. Cells were grown in liquid minimal medium prewarmed to a semirestrictive temperature (32 or 34°C) overnight and plated in 40 mM LiCl (16 mM for *cdc17*) in minimal medium agar, pH 5. Plates were incubated at the permissive temperature of 25°C for 5–7 d.

### Isolation of Chromosomal DNA and Pulsed-Field Gel Electrophoresis

Agarose-embedded chromosomal DNA was isolated essentially as described by Alfa *et al.* (1993). Cells from a 20-ml portion of a dense culture ( $A_{595} \sim 4$ ) were pelleted, washed in  $\text{H}_2\text{O}$ , and resuspended in  $\sim 0.3$  ml of buffer with lysing enzymes plus 0.4 ml of 1% agarose (Nusieve GTG agarose, FMC Bioproducts, Rockland, ME). The mixture was placed into plug molds (CHEF disposable plug molds, Bio-Rad, Richmond, CA). Before restriction enzyme digestion, the agarose plugs were rinsed twice in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and equilibrated with restriction enzyme digestion buffer (20–50 ml in 50-ml conical tubes). The plugs were loaded onto an agarose gel (ultrapure DNA-grade agarose, Bio-Rad), and electrophoresis was performed with a CHEF Mapper XA system (Bio-Rad). The electrophoresis conditions used in Figure 1 were 1% agarose, 0.5 $\times$  standard Tris-borate electrophoresis buffer, 120° included angle, 6 V/cm, 14°C. The switch time was 60 s for the first 15 h and 90 s for the next 9 h.

### Southern Hybridization

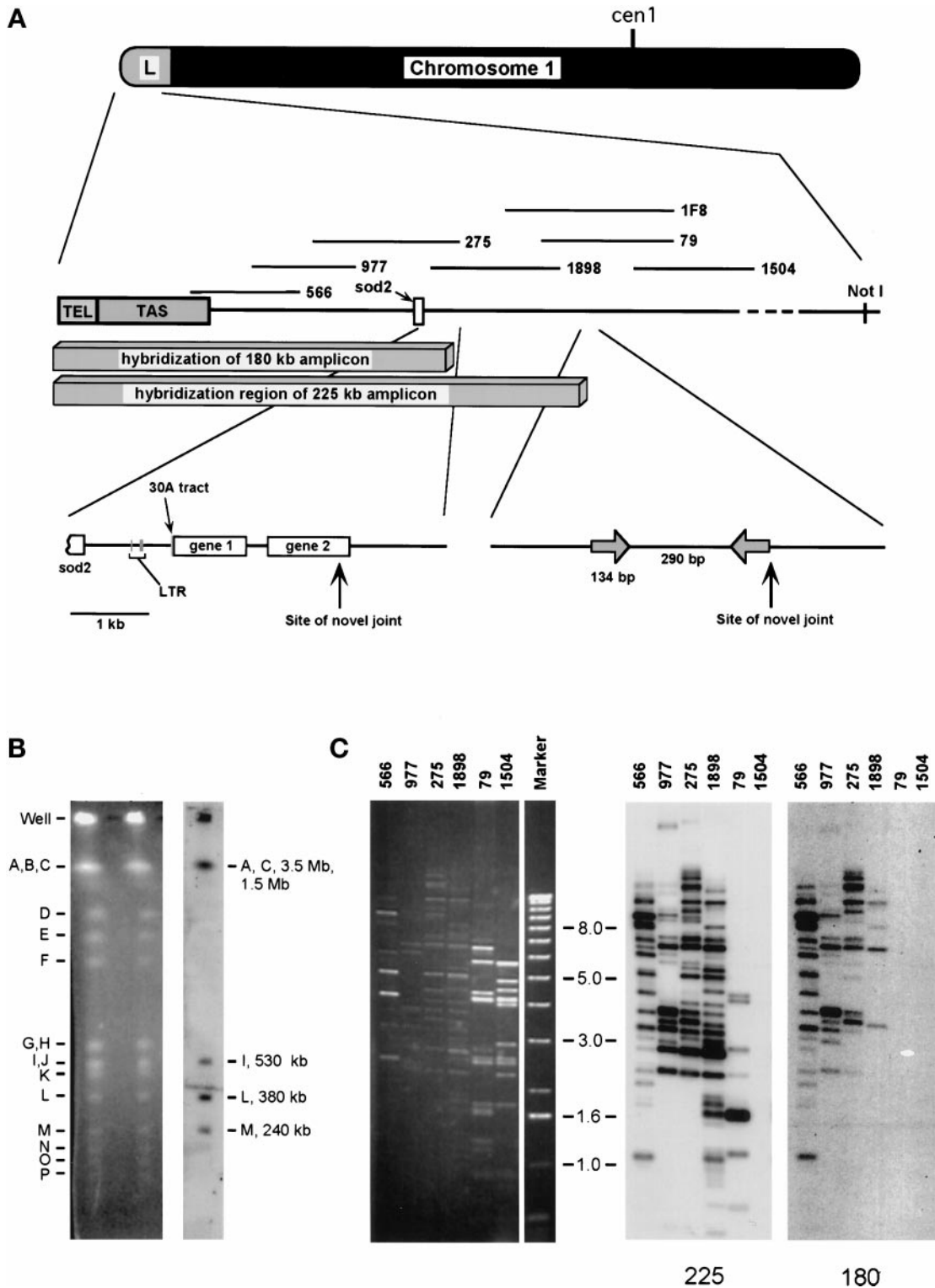
Probes were prepared with a random-primer-labeling kit (Mega-prime DNA-labeling system, Amersham, Arlington Heights, IL). To obtain amplicon DNAs, appropriate bands were cut from a pulsed-field gel, run as described above. The agarose was melted and diluted with sterile water to  $\sim 1$  ng/l, based on the amount of DNA (10–25 ng) estimated with ethidium bromide. Hybridizations were carried out in Church buffer (Church and Gilbert, 1984) at 65°C and washed with Church wash No. 2. Plasmids obtained from Neal Sugawara (laboratory of J. Szostak, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA) contained *S. pombe* telomeres or telomere-adjacent regions (Sugawara, 1988). The 800-bp *TaqI* fragment of plasmid pSPT16 was used as a probe to determine whether telomeric sequences were present in cosmids 566 and 977 (Mizukami *et al.*, 1993). A probe for the telomere-adjacent region was made from the entire plasmid pNSU64.

### Cloning and Sequencing

Transformations of competent *E. coli* (DH5) were performed with  $\text{CaCl}_2$ . The 1.7- and 3.2-kb *EcoRI*–*Bam*HI restriction fragments from cosmid 1898 were ligated into pBluescript II KS (+/–) and transformed by electroporation into Sure supercompetent *E. coli* (Stratagene, La Jolla, CA). Transformation of these plasmids, especially the 3.2-kb fragment, into DH5 cells produced no viable plasmid-containing clones. These regions were difficult to clone, possibly because of the presence of repeat sequences and secondary structures. However, we were able to recover clones of the 3.2-kb fragment when the plasmid was transformed into Sure cells. The sequence of the 1.7-kb fragment was obtained by cutting it into the following nonoverlapping fragments, which were subcloned into pBluescript and sequenced: *Bam*HI–*Xba*I, *Xba*I–*Kpn*I, *Kpn*I–*Eco*RV, *Eco*RV–*Hind*III (pBluescript was cut with *Hind*III–*Hinc*II), and *Hind*III–*Eco*RI. These plasmids were transformed into DH5 and sequenced from both ends with T7 and M13 reverse primers with the use of an ABI model 377 version 3.0 sequencer.

### Cloning of Amplicon Novel Joints

PCR was performed on DNA extracted from strains containing the 225-kb amplicon. The primers (5' to 3') were For 5 (GCTTTGCTAT-CATCGCCTAGC) and For 13 (ATACCTATACTTAGTTGCTAC), and the conditions were 59°C (or 63°C) annealing, 45-s extension, *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA). The first 180-kb amplicon novel joint was cloned with the use of a PCR-linker method. DNA from a strain carrying the 180-kb amplicon was digested with *Eco*RI, and the enzyme was inactivated by heating to 65°C for 15 min. The  $\sim 270$ -bp *Pvu*II–*Eco*RI fragment from Bluescript II KS (+/–), purified from agarose with Qiaex II (Qiagen, Chatsworth, CA), was used as the linker. The ligation reaction included the following: 0.25 ng/l linker, 5 ng/l digested genomic DNA, and  $\sim 1$  Weiss unit of T4 DNA ligase (Boehringer Mannheim). Properly ligated novel joints were recovered by PCR with the use of the pBluescript KS primer (CGAGGTCGACGG-TATCG), located within the linker and oriented toward the *Eco*RI site, and with For 3.2A (AGTTCGGTTTCCTATCTGCG), present in the 6.5-kb novel *Eco*RI fragment, also oriented toward the *Eco*RI site. After transformation into *E. coli*, two plasmids were isolated and sequenced. To obtain the two additional 180-kb amplicon novel joints, the primer R-180 (TTATTTAATGCTGAATAAACCTTCT), specific for DNA on the centromere-proximal side of the novel joint, was used with the primer For 3.2A in a standard PCR reaction (63°C annealing temperature) with the use of genomic DNA from two additional independent strains carrying the 180-kb amplicon. (The products of the PCR reaction with primers R-180 and For 3.2A were sequenced.) R-180 has only four mismatches compared with the genomic sequence of the 3.2-kb region. To confirm the specificity of this primer pair for the novel joint, rather than for the unrearranged



**Figure 1.** Hybridization of the 225- and 180-kb amplicons with cosmids representing the telomere-proximal third of *NotI* fragment L (~120 kb). (A) The subregion of *NotI* fragment L that hybridizes with the amplicons and characteristics of the amplified DNAs are shown. *NotI* fragment L is 380 kb long, and approximately one-third of it hybridizes to the amplicons. TEL, telomere; TAS, telomere-associated sequences; LTR, long terminal repeat. The cosmids, which are numbered, are drawn at their approximate locations. The *sod2* gene is depicted as a rectangle. The centromere-proximal 5 kb of hybridization between wild-type genomic DNA and the 180-kb amplicon contains *sod2*, a long

genomic sequence, the primer R-Gen (TTATTTAATGCTGAACA-GATCTTCA) was designed from the homologous sequence within the 3.2-kb *EcoRI*–*Bam*HI fragment of the *sod2* locus. When used with primer For 3.2A, primer R-180 amplified a 1.1-kb fragment from plasmids containing the novel joint, and primer R-Gen amplified a 1.1-kb fragment from plasmids containing the 3.2-kb fragment. No PCR product was obtained when the templates were placed in the opposite primer mix.

Sequences have been submitted to GenBank. The 180-kb amplicon-break-region genomic sequence has the accession number AF192974. The 180-kb amplicon novel joints (see Figure 6) have accession numbers AF207957, AF207958, and AF207959 for A, B, and C, respectively. The 225-kb amplicon novel joint (see Figure 4) has the accession number AF207956.

## RESULTS

### *Linear Extrachromosomal Amplicons Are the Predominant sod2 Amplification Product*

Selection with LiCl of wild-type *S. pombe* cells yielded 20 independent resistant strains. Three different amplification structures were observed by pulsed-field gel electrophoresis. As found previously (Patterson *et al.*, 1999), the frequency of resistance was  $\sim 10^{-6}$  per cell per generation. Extrachromosomal amplicons were predominant, but an intrachromosomal amplicon was observed in one case. Linear extrachromosomal amplicons of 225 or 180 kb have been reported (Patterson *et al.*, 1999). All 20 strains contained either the 225-kb amplicon (15 of 20) or the 180-kb amplicon (5 of 20). No strain contained both the 225- and 180-kb amplicons. Three strains contained megabase-long extrachromosomal amplicons in addition to a 225- or 180-kb linear extrachromosomal amplicon. One of these amplicons migrated differently relative to size markers depending on the switch time during pulsed-field gel electrophoresis (our unpublished results), suggesting a circular structure. One strain appeared to have *sod2* amplified intrachromosomally as well as extrachromosomally. In this strain, *NotI* fragment L (380 kb), which contains the endogenous *sod2* gene, was absent and was replaced by a new band of 610 kb (our unpublished results). We have studied in detail the two most frequently observed extrachromosomal linear amplicons.

### *The 225- and 180-kb Amplicons Both Contain Genomic DNA from the Telomere-proximal Third of NotI Fragment L*

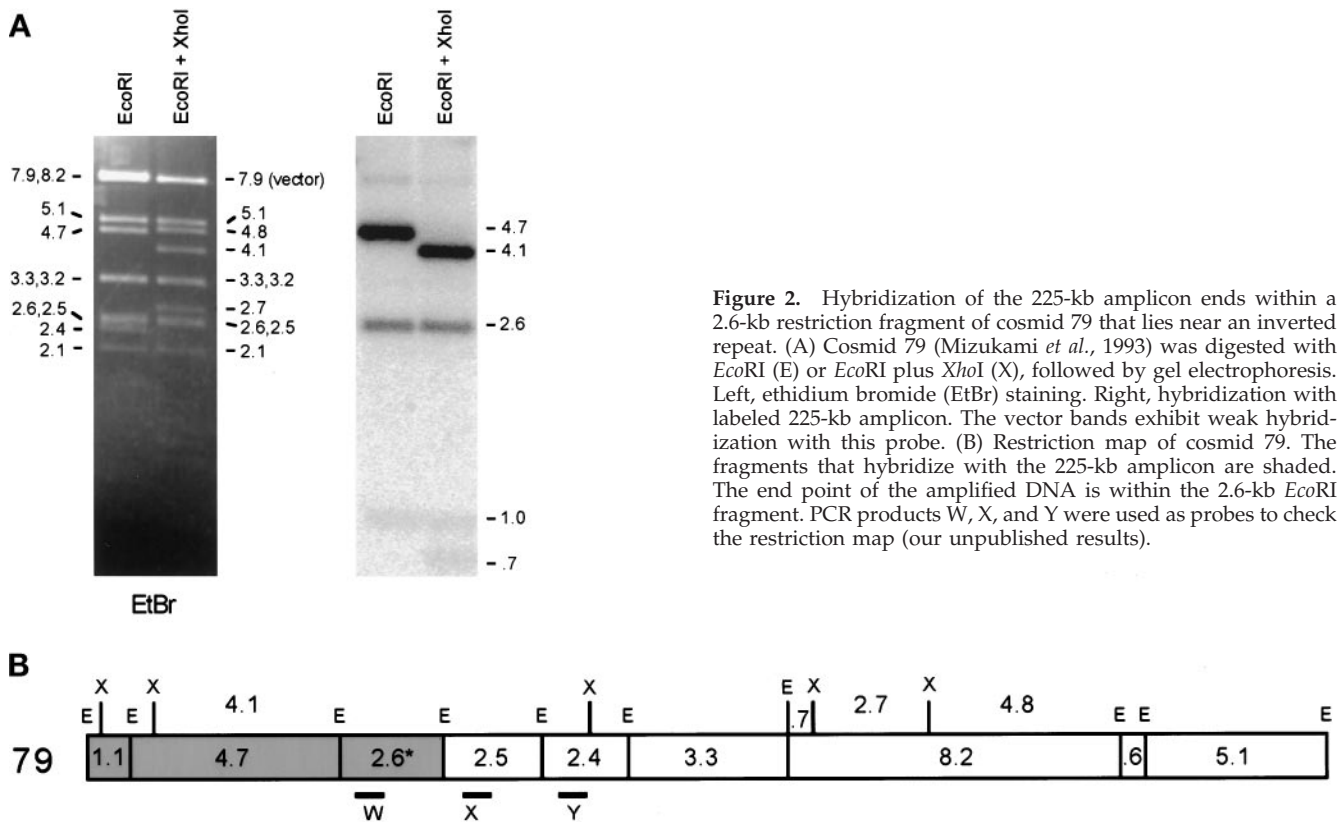
Figure 1A shows an overview of the region of chromosome I contained in the linear amplicons and of cosmids covering the region. To determine the origin of the amplified DNA, a Southern blot of *NotI*-digested wild-type genomic DNA was

hybridized with gel-purified, radiolabeled amplicon DNA. Probes from both amplicons hybridized with the highest intensity to fragment L, but they also hybridized to the other telomeric fragments (A, C, I, and M) (data for the 225-kb amplicon are shown in Figure 1B; data for the 180-kb amplicon are not shown). *NotI* fragment L (380 kb) represents the end of the long arm of chromosome I and contains both the telomere and *sod2* (Fan *et al.*, 1989; Patterson *et al.*, 1999). The amplicons contain telomeric DNA (Patterson *et al.*, 1999), and both of the amplicon probes hybridized with the five telomeric *NotI* fragments (chromosome III is not cut by *NotI*) and the telomeric *SfiI* fragments. We noted that both the amplicons were smaller than *NotI* fragment L.

Cosmids from the Hoheisel library (Hoheisel *et al.*, 1993) representing *NotI* fragment L plus cosmids from the Mizukami library (Mizukami *et al.*, 1993) near the telomere were used. The Mizukami library represents more of the telomeric end of *NotI* fragment L. Cosmids from the latter library are shown in Figure 1C. Southern hybridization of cosmids covering *NotI* fragment L indicated that the 225-kb amplicon hybridizes with the region covered by cosmids 566 (the cosmid closest to the telomere), 977, 275, 1898, and 79, altogether  $\sim 120$  kb of fragment L. The 180-kb amplicon hybridized to cosmids 566, 977, 275, and 1898, a region of  $\sim 80$  kb. These estimations are based on cosmid insert sizes plus  $\sim 10$  kb of telomere-adjacent sequences. Cosmid 566 does hybridize with telomere-adjacent sequences but does not hybridize with true telomeric sequences (our unpublished results). Thus, the insert of cosmid 566 extends into the telomere-adjacent region, but the distance between the end of the insert and the end of the chromosome is not known. The telomere-adjacent DNA has been estimated to be  $>19$  kb long (Sugawara, 1988). We estimate that 100–130 kb of chromosome I DNA is represented in the 225-kb amplicon and that 70–90 kb of chromosome I DNA is represented in the 180-kb amplicon. These sizes are consistent with amplicons that each contain two copies of the region.

To locate the end point of the genomic region contained in the 225-kb amplicon, we performed Southern hybridization experiments with restriction digests of cosmid 79 (Mizukami *et al.*, 1993). Only the telomere-proximal 8.4 kb (the 1.1-, 4.7-, and 2.6-kb *EcoRI* fragments) of the cosmid was present (Figure 2). Two fragments comigrated at 2.6 kb, designated 2.5 and 2.6 in Figure 2A. The sequence of cosmid 1F8 (Hoheisel *et al.*, 1993; GenBank No. Z81312) (Figure 1A) predicts a 3.0-kb *EcoRI* fragment. Inspection of the sequence reveals a 500-bp direct repeat within this 3.0-kb fragment. We believe that this duplication is an artifact of sequencing and is not present in either the cosmid or the genomic sequence. A restriction map based on our data is shown in Figure 2B, with a 2.6-kb fragment in place of the 3.0-kb fragment. Use

**Figure 1 (opposite)** terminal repeat sequence, a stretch of 30 adenosine residues, and two ORFs. The centromere-proximal 1 kb of hybridization between wild-type genomic DNA and the 225-kb amplicon contains a 134-bp inverted repeat and a 290-bp spacer. (B) Wild-type chromosomal DNA digested with *NotI*. The digest was separated by pulsed-field gel electrophoresis. Left, ethidium bromide-stained gel. Right, hybridization with labeled 225-kb amplicon. *NotI* fragment L contains *sod2* and a telomere. *NotI* fragments C, I, and M all contain telomeres. A indicates intact chromosome III. *NotI* fragments C and A do not separate well under the running conditions used in this experiment. (C) Cosmids from the Mizukami library (Mizukami *et al.*, 1993) digested with *HindIII*, with the most telomere-proximal cosmid on the left. Left, ethidium bromide staining. Right, duplicate transfers hybridized with the 225- or 180-kb amplicon. All of *NotI* fragment L was tested, and only cosmids in the region shown hybridized. DNA Marker X (Boehringer Mannheim) was used for size markers.



**Figure 2.** Hybridization of the 225-kb amplicon ends within a 2.6-kb restriction fragment of cosmid 79 that lies near an inverted repeat. (A) Cosmid 79 (Mizukami *et al.*, 1993) was digested with *EcoRI* (E) or *EcoRI* plus *XhoI* (X), followed by gel electrophoresis. Left, ethidium bromide (EtBr) staining. Right, hybridization with labeled 225-kb amplicon. The vector bands exhibit weak hybridization with this probe. (B) Restriction map of cosmid 79. The fragments that hybridize with the 225-kb amplicon are shaded. The end point of the amplified DNA is within the 2.6-kb *EcoRI* fragment. PCR products W, X, and Y were used as probes to check the restriction map (our unpublished results).

of PCR probes from each of the *EcoRI* fragments in the area (Figure 2B, W, X, and Y) showed that, as expected, sequences in the 2.5- and 2.4-kb fragments were not in the 225-kb amplicon (our unpublished results) and that sequences from the 2.6-kb fragment were present in the amplicon. Therefore, the 2.6-kb *EcoRI* fragment contains the boundary of the genomic DNA duplicated in the 225-kb amplicon.

#### **The Novel Joint of the 225-kb Amplicon Contains an Inverted Repeat**

The 2.6-kb *EcoRI* fragment of cosmid 1F8 hybridizes with novel restriction fragments of the 225-kb amplicon (Figure 3). We conclude that the 225-kb amplicon extends into and stops within the sequence of this 2.6-kb *EcoRI* fragment at a novel joint where DNA from one region of the genome joins DNA from another region. The 2.6-kb fragment includes two exact copies of a 134-bp sequence in an inverted orientation, separated by a 290-bp spacer. Beyond the ends of the 134-bp elements, their similarity declines rapidly. The inverted repeat is homologous to the long terminal repeat (LTR) of Tf2 and Tf1-107 retrotransposons (GenBank Nos. L10324 and M38526) but contains only a portion of the LTR sequence and is not an intact retrotransposon. We used the genomic 2.6-kb *EcoRI* fragment to identify novel fragments from the 225-kb amplicon. The sizes of these novel fragments (Figure 3A) support the interpretation that the palindrome extends for at least 4 kb on either side of the original inverted repeat. The presence of two amplified *SnaBI* fragments (3.9 and 4.0

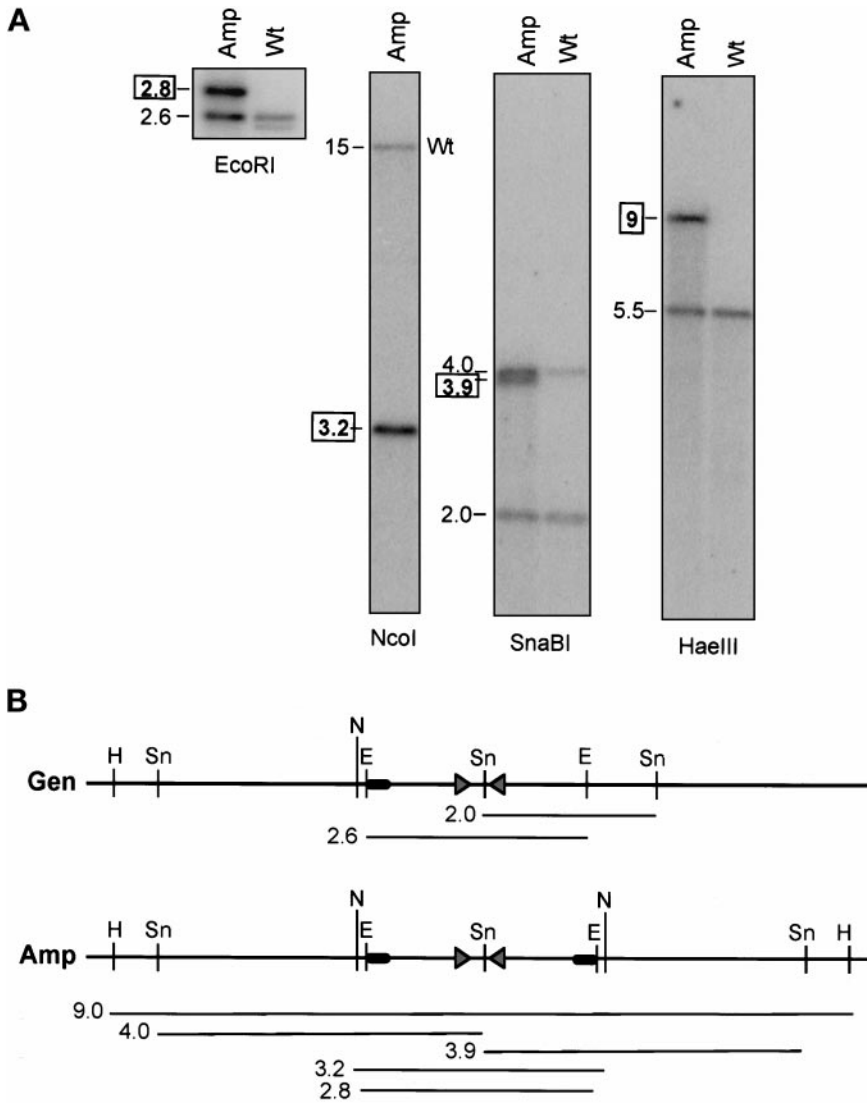
kb) of approximately equal intensity indicates that the 290-bp spacer, which contains a *SnaBI* site, has been conserved in the amplicon. The wild-type restriction fragments appear to be conserved.

#### **Sequence of the Novel Joint of the 225-kb Amplicon**

PCR was used to clone the novel joint (Figure 4A). Primer For 5 binds on the telomeric side of the inverted repeats and is oriented to prime DNA synthesis toward them. Primer For 13 lies within the 290-bp spacer, which is oriented in the same direction as primer For 5. These primers cannot amplify a fragment from wild-type DNA, but they can amplify a fragment from a sequence that is palindromic around the inverted repeats. As expected, no PCR product was obtained when these two primers were used with wild-type DNA, but a fragment of ~650 bp was produced when the DNA from a strain containing a 225-kb amplicon was used as a template (Figure 4B). The 650-bp For 5/For 13 PCR products from four independent amplicons were identical and contained the spacer, one repeat element, and DNA upstream of the inverted repeat in an inverted orientation, as expected for a palindromic novel sequence (Figure 4C; GenBank No. AF207956).

#### **The Novel Joint of the 180-kb Amplicon Is Not Formed at an Inverted Repeat**

To locate the end of the genomic region in the 180-kb amplicon, we performed Southern hybridization experiments

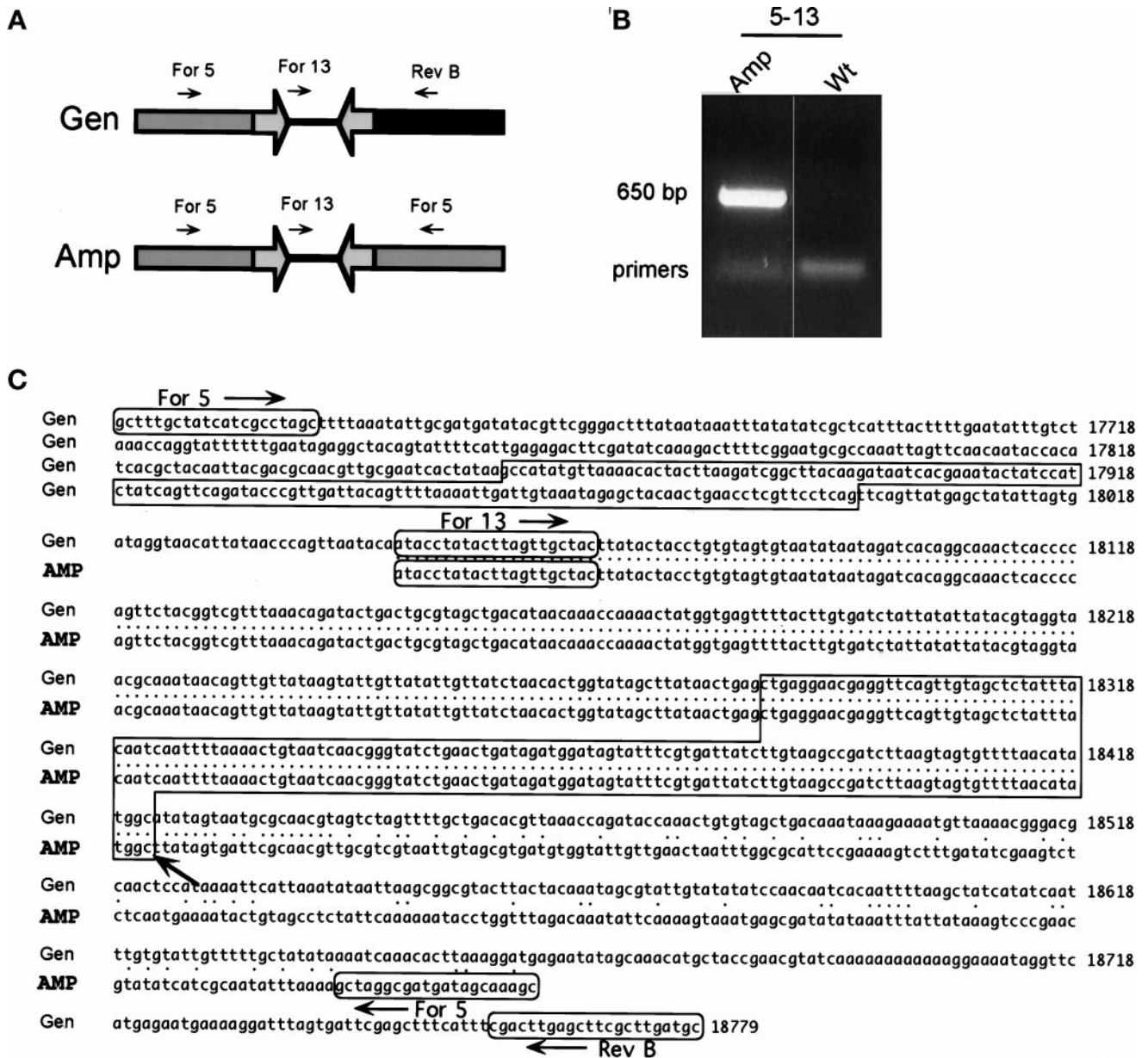


**Figure 3.** Novel restriction fragments detected in the 225-kb amplicon by the 2.6-kb *EcoRI* fragment. (A) Sheared genomic DNA from the wild-type (Wt) strain and from a strain carrying a 225-kb amplicon (Amp) was digested with *EcoRI*, *SnaBI*, *HaeIII*, or *NcoI*, and the fragments were separated by gel electrophoresis. The transfers were hybridized with labeled PCR products from within the 2.6-kb *EcoRI* fragment of cosmid 79. Numbers indicate the sizes of the fragments in kilobases. Boxed numbers indicate novel fragments, which were detected only in strains containing the 225-kb amplicon. (B) Restriction map of the novel fragment. Gen, map of the genomic region. Amp, map of the amplicon, deduced from the sizes of the novel fragments. The map of chromosome I is deduced from sequence data (the *S. pombe* genome project) and restriction data (our unpublished results). The sizes of the novel fragments are consistent with a palindromic amplicon with a novel joint immediately after the inverted repeat. The triangles represent the inverted repeats. The black ovals represent unique sequences found only telomere-proximal to the inverted repeats in wild-type DNA. E, *EcoRI*; Sn, *SnaBI*; H, *HaeIII*; N, *NcoI*

with restriction digests of cosmid 1898 (Mizukami *et al.*, 1993). Only the telomere-proximal 4.9 kb of this cosmid is present in the 180-kb amplicon (Figure 5). The 4.9-kb genomic sequence was submitted to GenBank (No. AF192974). Note that the 4.9- and 4.7-kb fragments are not resolved in this figure, but the 4.7-kb fragment is a part of cosmid 79, which does not hybridize to the 180-kb amplicon (Figure 1C). Additional experiments (our unpublished results) narrow the end point region to within the 3.2-kb *BamHI*-*EcoRI* fragment, which was therefore used to probe a Southern transfer of genomic DNA from amplicon-containing strains. One amplified novel fragment and two wild-type fragments were observed per digest (Figure 6A). Notably, the size of the *EcoRI* novel fragment is ~6.5 kb, whereas the wild-type fragments are 8 and 12–15 kb long. The native 8-kb fragment is from chromosome I, whereas the 12- to 15-kb fragment appears to be from chromosome II (our unpublished results). The restriction map of the amplicon is derived from the sizes of

novel fragments compared with those of the chromosomal fragments (Figure 6B). We conclude that the 180-kb amplicon is not a palindrome.

The 1.7- and 3.2-kb *EcoRI*-*BamHI* fragments in the region of the novel joint were subcloned from cosmid 1898 into pBluescript II KS and sequenced. The first 375 bp of cosmid 1898 are identical to the 3'-untranslated region of a cDNA plasmid containing the *sod2* gene, the precise genome location of which was previously unknown. The sequence of cosmid 1898 begins ~284 bp downstream of the stop codon of *sod2*. A run of 30 contiguous adenosine residues, an unusual sequence in *S. pombe* DNA, begins at position 1361 of cosmid 1898. Retrotransposon sequences were found within this region of the 1.7-kb fragment; interestingly, this is the same portion of the LTR sequence that constituted the inverted repeat associated with the novel joint of the 225-kb amplicon. There are several large ORFs within the 4.9-kb sequence represented in Figure 1A. The first has high similarity to *L-asparaginase* (*L-asp*)

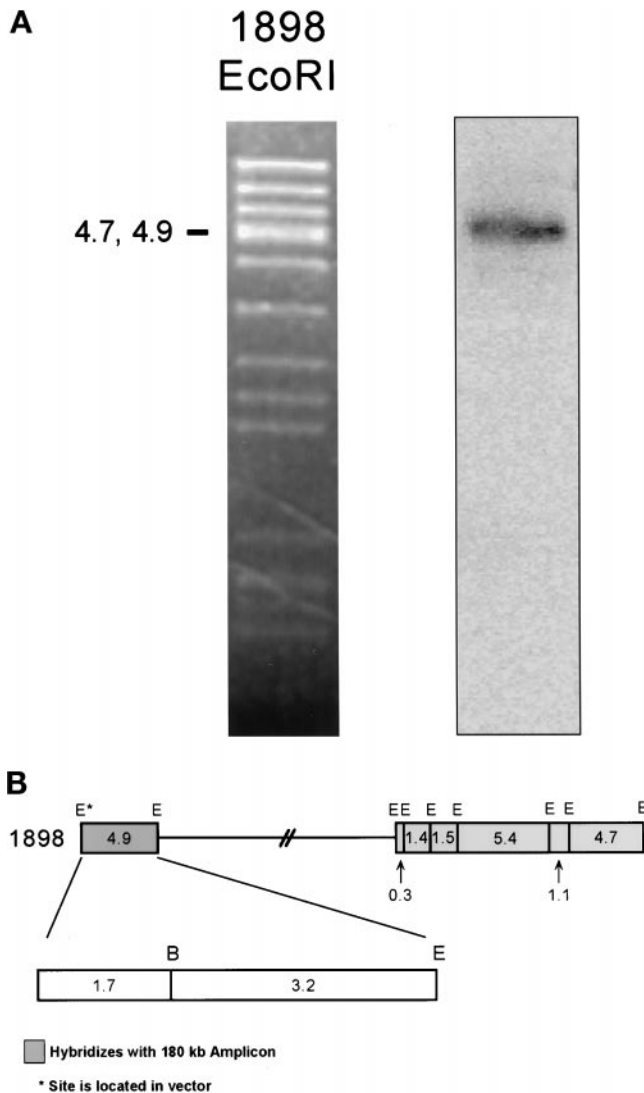


**Figure 4.** Sequence of the novel joint of the 225-kb amplicon. (A) The PCR strategy used to clone the novel joint. The proposed structure of the amplicon is shown, together with the PCR primers For 5, For 13, and Rev B. Open arrows represent the inverted repeat. The primer pair For 5–For 13 together can produce a PCR product only if an inversion of one of the primers has occurred. The primer pairs For 5–Rev B and For 13–Rev B can produce products only from wild-type DNA. (B) The PCR products obtained with primers For 5 and For 13. Total DNA isolated from the wild-type (Wt) strain and from strains containing the 225-kb amplicon were used as templates for PCR. The 650-bp PCR product For 5–For 13 was observed only with template DNA from strains carrying the 225-kb amplicon. The other PCR products were observed with all templates tested, as expected (our unpublished results). (C) The sequence of the novel joint. Gen, sequence of wild-type chromosome I. Amp, sequence of the 650-bp PCR product shown in B. The inverted repeats are boxed, and the novel joint lies precisely at the end of the centromere-proximal repeat (arrow). Four independent 225-kb novel joints were sequenced, and all were identical (GenBank No. AF207956).

(GenBank No. Y11944), which is near the telomere of the short arm of chromosome I (Bonthon, personal communication). Both ORFs are present in the expressed sequence tag database, indicating that they are expressed genes (GenBank Nos. AU013226 and AU009847).

#### *The 180-kb Amplicon Is Formed as a Result of an Interchromosomal Event*

The sequences of novel joints from three independent 180-kb amplicons were very similar. They have been submitted to



**Figure 5.** Hybridization of the 180-kb amplicon ends within the 3.2-kb *Bam*HI–*Eco*RI restriction fragment of cosmid 1898. (A) Cosmid 1898 (Mizukami *et al.*, 1993) was digested with *Eco*RI, and the fragments were separated by gel electrophoresis. Left, ethidium bromide–stained gel. Right, hybridization with labeled 180-kb amplicon. Only the 4.9-kb *Eco*RI restriction fragment hybridized. (B) Partial restriction map of cosmid 1898. Hybridization data from A are shown in relationship to the restriction map. The end point of the amplified DNA lies within the 4.9-kb *Eco*RI fragment. Additional data (our unpublished results) localize the end point to within the 3.2-kb *Bam*HI–*Eco*RI fragment. E, *Eco*RI; B, *Bam*HI.

GenBank (Nos. AF207957, AF207958, and AF207959). The sequence centromere-proximal to the novel joint shares approximately the same degree of similarity with the *L-asp* sequence as does the 4.9-kb *Eco*RI sequence from cosmid 1898. The sequence centromere-proximal to the novel joint is not present in the expressed sequence tag database. Alignment of the novel joints with the 4.9-kb *Eco*RI fragment of cosmid 1898 (Figure 6C) reveals a pattern of nucleotide changes consistent with a mechanism involving homolo-

gous recombination between the two sequences homologous to *L-asp*. Examination of these sequences reveals that the novel joints have occurred by bp 3534. The sequence of C between the stars (Figure 6C) was not obtained, but the lack of this sequence does not detract from the above conclusions. The observations are consistent with recombination occurring at one site within this region of cosmid 1898.

To determine the chromosomal origin of the sequence on the centromeric side of the novel joint, the 320-bp *Eco*RV fragment from plasmid 1.1D (corresponding to the terminal 320 bp of the 1.1-kb novel joint sequence) was used to probe a pulsed-field gel electrophoresis transfer containing *Not*I restriction fragments from wild-type *S. pombe* DNA (Figure 7A). Fragments L (the *sod2*-containing telomeric fragment from chromosome I) and M (the telomeric fragment from the short arm of chromosome II) hybridized with the probe. The result was confirmed with a smaller probe from the centromere-proximal sequence of the novel joint, which hybridized only to *Not*I fragment M (our unpublished results). We conclude that the 180-kb amplicon is formed by homologous mitotic recombination between the transcribed *L-asp* homologue located ~5 kb away from the *sod2* gene on the centromeric side (and ~70 kb from the telomere) and another *L-asp* homologue located on the short arm of chromosome II, ~110 kb from the telomere. The *L-asp* homologue on chromosome II apparently is oriented with the direction of transcription toward the telomere.

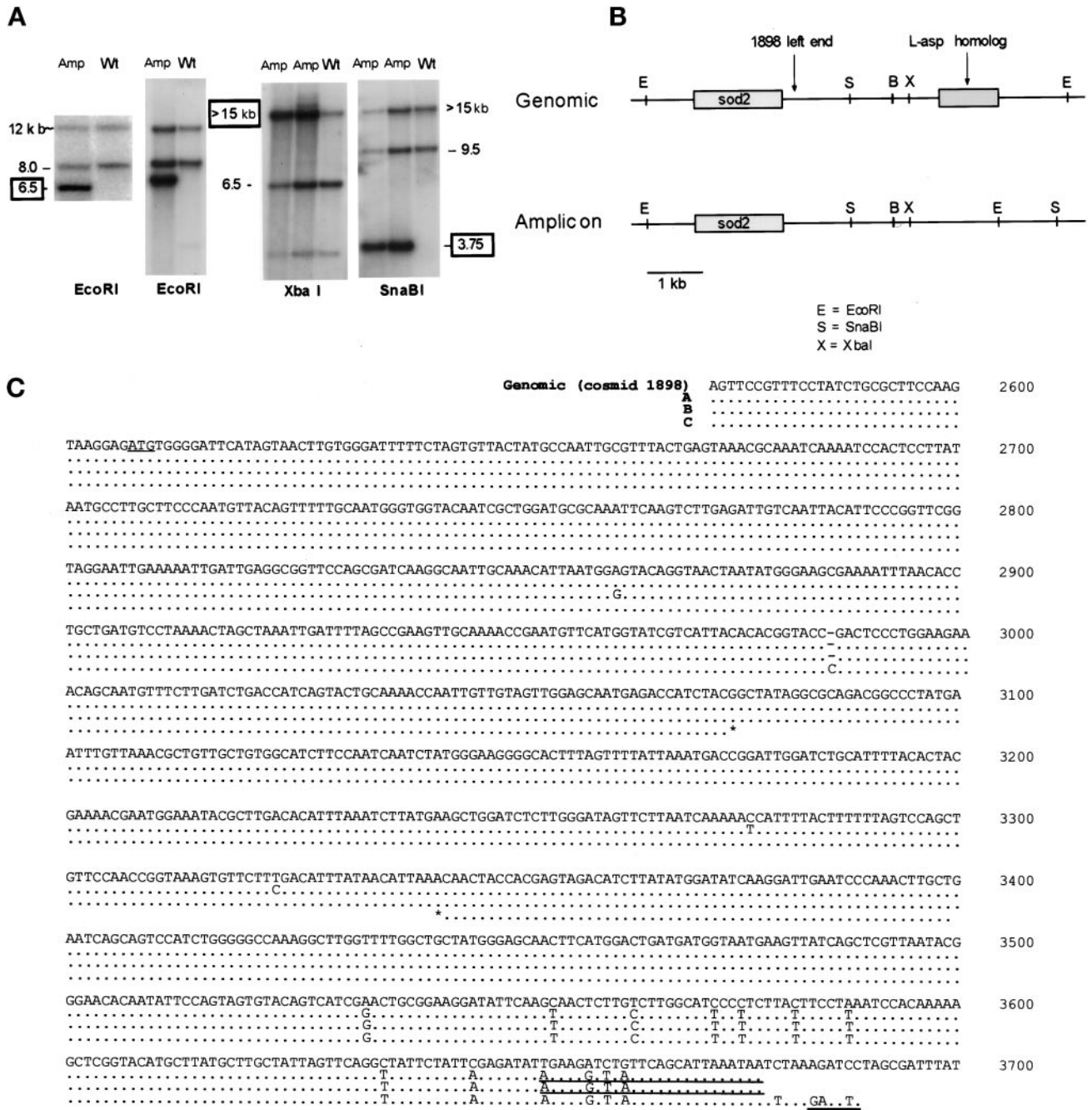
### Topoisomerase and Ligase Mutations Increase the Frequency of *sod2* Amplification

Mutations increasing the probability of DNA breakage in a cell may affect the frequency of DNA amplification. Taking mutations in DNA ligase and topoisomerase as examples, we determined *sod2* amplification frequencies in haploid strains bearing temperature-sensitive alleles of these genes at a semirestrictive temperature (Nasmyth, 1979) (Table 1). In *S. pombe*, both *top1* and *top2* have type I topoisomerase activity (Uemura and Yanagida, 1984). Consequently, a substantial decrease in topoisomerase I activity is observed only in the *top1 top2* double mutant. A *top1 top2* strain had a 200-fold increase in amplification frequency, and a *top2(mut)* strain with a normal *top1* gene had only a 3-fold increase. A strain with a temperature-sensitive mutation in DNA ligase (*cdc17*) exhibited a 600-fold increase in amplification frequency at the semirestrictive temperature compared with the wild-type strain. Both size classes of amplicon were observed in the mutant strains at about the same ratio as in wild-type cells (our unpublished results). In 10 ligase-deficient *sod2*-amplified strains, nine 225-kb amplicons and one 180-kb amplicon were observed. In 12 *top1 top2*-deficient strains amplified for *sod2*, nine 225-kb amplicons and three 180-kb amplicons were observed.

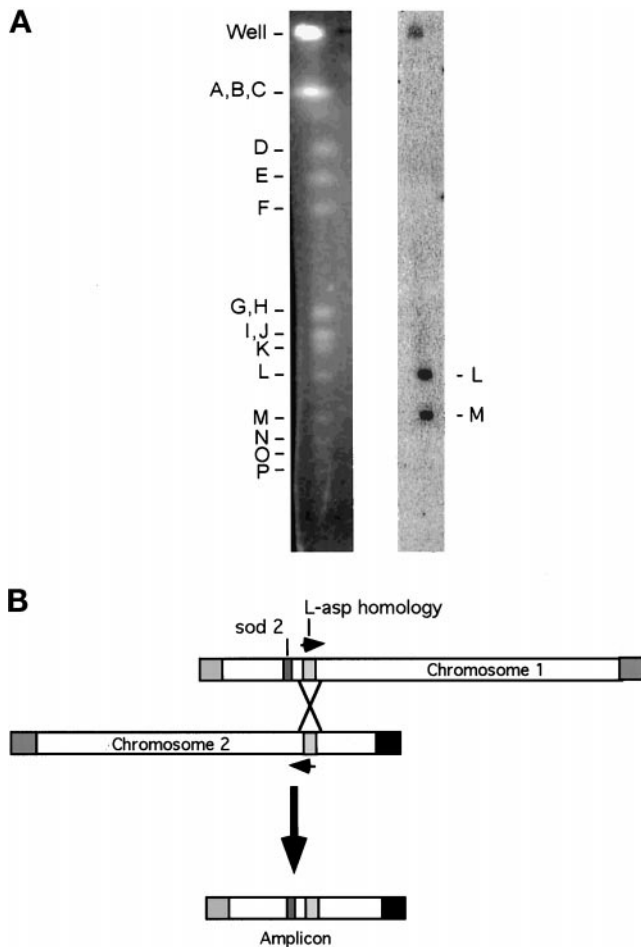
## DISCUSSION

Identification of the novel joint sequences for each class of amplicon characterized was essential for a molecular characterization of the amplicons. The novel joints and structure were different between the two amplicon classes. Each LiCl-resistant strain studied contained a *sod2* linear extrachromo-





**Figure 6.** A novel restriction fragment in the 180-kb amplicon. (A) Sheared genomic DNA from the wild-type (Wt) strain and from two strains carrying 180-kb amplicons (Amp) was digested with *EcoRI*, *XbaI*, or *SnaBI*, and the fragments were separated by gel electrophoresis. The transfers were hybridized with the 3.2-kb *BamHI-EcoRI* fragment of cosmid 1898. The numbers indicate sizes in kilobases. Boxed numbers indicate novel fragments detected only in strains containing the amplicon. (B) Restriction map of the novel fragment. The partial map of chromosome I is derived from our sequence and restriction data (our unpublished results). The 4.9-kb genomic fragment sequence has been submitted to GenBank (No. AF192974). (C) Sequence of the 1.1-kb novel joint PCR products from the 180-kb amplicon. The novel joint sequences have been submitted to GenBank (Nos. AF207957, AF207958, and AF207959 for A, B, and C, respectively). The region to the left (telomere-proximal) of the site of recombination is identical to the 3.2-kb *BamHI-EcoRI* fragment from cosmid 1898. Numbers correlate to the sequence of cosmid 1898 beginning at the telomere-proximal end. The 150 bp of sequence to the right (centromere-proximal) of the novel joints abuts the novel *EcoRI* site. Genomic indicates wild-type genomic DNA represented by cosmid 1898 sequence. A, B, and C represent three independent sequences. The recombination has clearly occurred in all three amplicons by bp 3534. Differences from the genomic sequence before this are believed to be *Taq*-induced errors. Only portions of PCR product C outside the stars were sequenced with fidelity. The underlined ATG represents the putative start site of the *L-asp* homologue, the longest ORF. Also underlined is the binding site of primer R-180 and the novel *EcoRI* site.



**Figure 7.** Hybridization of the 180-kb novel joint region with *NotI* fragments L and M. (A) Wild-type *S. pombe* DNA, digested with *NotI*, was separated by pulsed-field gel electrophoresis. Left, ethidium bromide-stained gel. Right, hybridization with novel joint region fragment. The 320-bp novel joint region fragment was an *EcoRV* fragment of plasmid 1.1D. The experiment was repeated with a smaller probe from the centromere-proximal sequence of the novel joint, which hybridized only to *NotI* fragment M (our unpublished results). (B) A homologous recombination model for the 180-kb amplicon. The *L-asp* homologue on chromosome I is oriented with the direction of transcription away from the telomere (our unpublished results). *sod2* is represented as a dark gray rectangle. The *L-asp* homology region is represented as a light gray rectangle. The arrows denote the direction of transcription of the *L-asp* homologues. The squares represent the telomeres: the chromosome I long arm telomere is gray and the chromosome II short arm telomere is black. The horizontally striped box is the telomere of the short arm of chromosome I, and the vertically striped box is the telomere of the long arm of chromosome II.

somal amplicon of 180 kb (25%) or 225 kb (75%). The 225-kb amplicon is a palindrome of a region bounded by an inverted repeat and a telomere. The 180-kb linear amplicon results from the fusion of two chromosome ends through a region of homology.

The novel joint of the 180-kb amplicon occurs within homologues of *L-asp* located near the telomeres of the long arm of chromosome I and the short arm of chromosome II,

**Table 1.** Increases in *sod2* amplification in *S. pombe* strains with temperature-sensitive mutations in the ligase and topoisomerase genes

Strain	Temperature (°C)	Hours at temperature shown	Viability (%)	Fold increase in amplification frequency <sup>a</sup>
<i>cdc-17</i> (ligase)	25 <sup>b</sup>	16	100	6
<i>cdc-17</i> (ligase)	34 <sup>c</sup>	16	10	600
<i>top1 top2</i>	25 <sup>b</sup>	16	100	3
<i>top1 top2</i>	34 <sup>c</sup>	16	20	200
<i>top2</i>	25 <sup>b</sup>	16	100	1
<i>top2</i>	32 <sup>c</sup>	7	5	3

Cells grown for the times and at the temperatures shown were pelleted, resuspended, and plated at  $5 \times 10^7$  cells per selection plate. The plates were incubated at the permissive temperature of 25°C for 5–7 d before scoring of LiCl-resistant colonies.

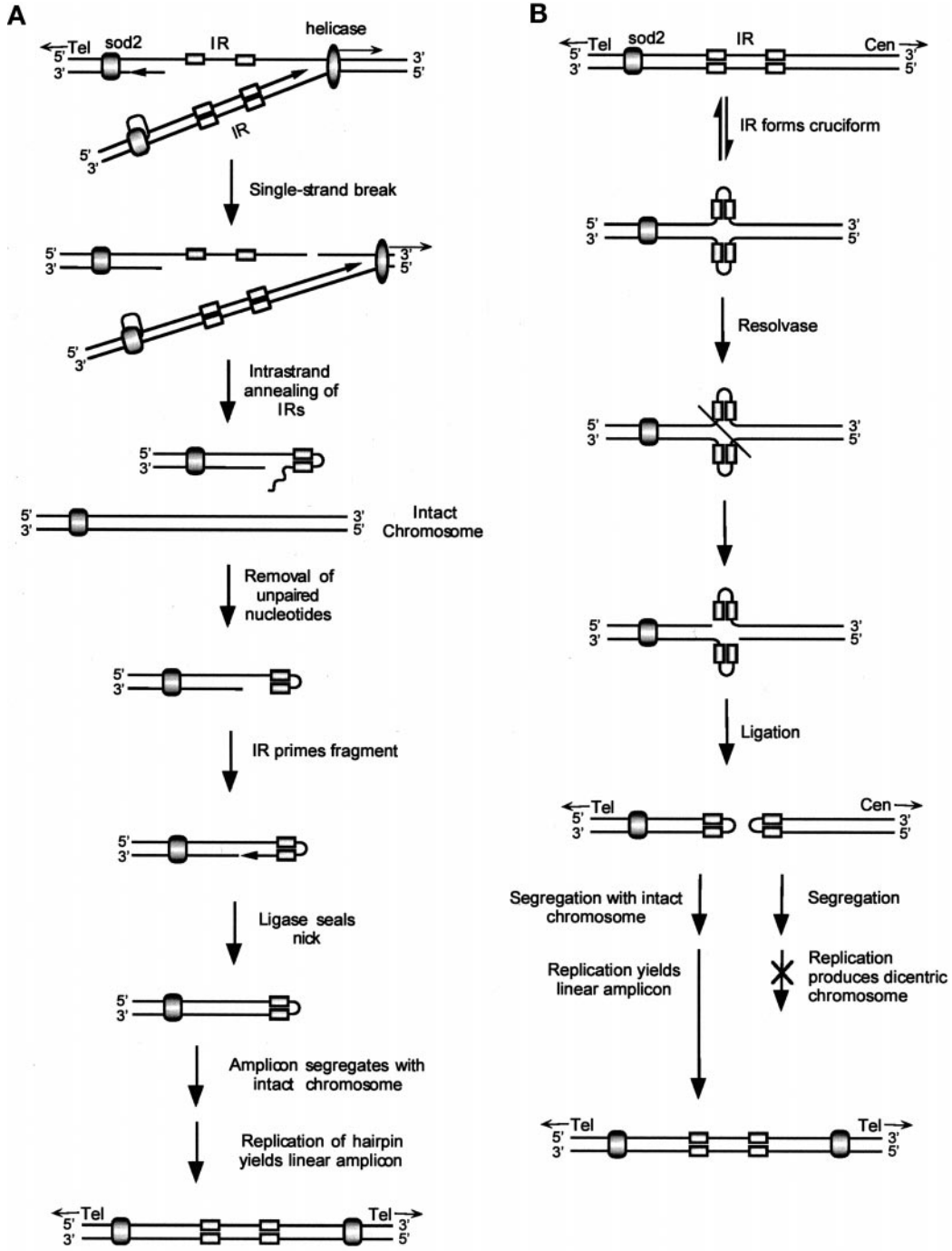
<sup>a</sup> Compared with wild type and corrected for viability.

<sup>b</sup> Permissive temperature.

<sup>c</sup> Semirestrictive temperature.

indicating recombination between these two homologous regions (Figure 7B). Because the amplified strains studied have intact chromosomes I and II and are haploid, we conclude that this recombination event must occur after DNA replication. The event must be mitotic because the cells do not enter meiosis during selection. Additional cell cycles can generate multiple copies of the amplicon. Similar recombination-based mechanisms of gene amplification that use repeats of homologous regions have been proposed (Edlund and Normark, 1981; Whoriskey *et al.*, 1987; Wu *et al.*, 1991; Grondin *et al.*, 1993). In *E. coli* and phage T4, head-to-tail arrays of 50–100 copies are generated when the amplified DNA is flanked by short (<20 bp) direct repeats (Whoriskey *et al.*, 1987; Wu and Black, 1987). In *Leishmania*, amplicons of the P-glycoprotein gene are extrachromosomal circles containing two head-to-tail copies of the region and are formed with the participation of 541-bp direct repeats (Grondin *et al.*, 1993, 1996). In the amplification of *sod2* in *S. pombe*, a long run of homology is involved, similar to the situation in *Leishmania*. When the full genomic sequence for this region of *S. pombe* becomes available, the length of the homologous region will be revealed.

The novel joint of the 225-kb amplicon occurs precisely at the end of an exact inverted repeat, arguing for an essential role of the repeat in amplicon formation. An aberrant replication model (Figure 8A) invokes a single-strand break in the DNA downstream of both the inverted repeat and the replication fork, allowing the inverted repeat to form a hairpin. After the unpaired nucleotides are removed, the inverted repeat primes replication of an additional DNA synthesis fragment, and DNA ligase seals the nick. A large hairpin duplication of the left end of the chromosome results, and a telomere-deleted chromosome is a by-product. The hairpin segregates together with an intact chromosome I to generate a viable cell with a small selective advantage. An additional cell cycle is required to form the mature double-stranded amplicon. Additional cycles yield cells



**Figure 8.** Replication and recombination models for the 225-kb amplicon. (A) Replication model. *sod2* is flanked by a telomere (Tel) on the left and an inverted repeat (IR) on the right. *sod2* is shown as a shaded oval, the inverted repeats are shown as rectangles, and the helicase/replication complex is shown as an elongated oval. A single-strand DNA break on the lagging strand, to the right of the inverted repeat and ahead of the replication fork, allows the inverted repeat to form a hairpin. Replication of the other DNA strand is unaffected. Removal of unpaired nucleotides allows the hairpin to prime DNA synthesis. After ligase seals the nick, a larger hairpin, consisting of a duplication of the region from the inverted repeat to the telomere, is formed. The large hairpin and intact chromosome I must segregate together to yield a viable cell resistant to LiCl. The mature palindrome is formed in the next cell cycle. (B) Recombination model. The inverted repeat to the right of *sod2* forms a cruciform analogous to a Holliday structure. If resolvase cuts across the axis of symmetry and ligase joins the free ends of the different DNA strands, two hairpins will be produced that include *sod2* and the centromere (Cen), respectively. The *sod2* hairpin must segregate with intact chromosome I to form a viable cell resistant to LiCl. The next round of replication produces the mature palindrome. Because the fission yeast *S. pombe* is haploid during selection with LiCl, this mitotic recombination event must occur after replication of the DNA.

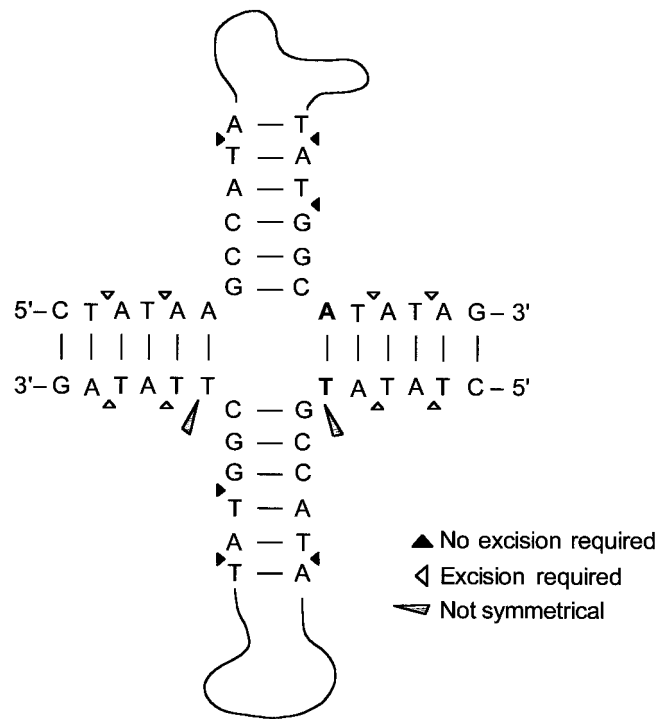
with multiple copies of the amplicon and a correspondingly increased growth advantage in selective medium.

Similar models have been presented for both *Tetrahymena* and *Leishmania* (Ford *et al.*, 1985; Yasuda and Yao, 1991; Butler *et al.*, 1996; Grondin *et al.*, 1996). When the *Leishmania* P-glycoprotein locus is flanked by inverted repeats, it forms an inverted duplication upon amplification (Grondin *et al.*, 1996). White *et al.* (1988) suggested that annealing of these repeats during a block in replication could serve as a primer for DNA polymerase. The newly synthesized strand could then serve as a template to generate a large inverted duplication. Others (Ouellette *et al.*, 1991; Cohen *et al.*, 1994) have suggested that stalling of the replication fork would allow the inverted repeats to anneal, enabling strand switching, or "U-turn" replication. Our model differs in that formation of the hairpin does not require a single strand to invade duplex DNA. For our model to produce both a palindromic amplicon and an intact chromosome, a single-strand DNA break is required between the inverted repeat and the centromere. A double-strand break, causing stalling of replication and switching of both strands, is incompatible with maintaining an intact chromosome.

An alternative recombination-resolvase model (Figure 8B) uses a recombination event that leads to a large hairpin precursor of the amplicon. This model, which is very similar to those proposed previously for *Tetrahymena thermophila* (Yasuda and Yao, 1991; Butler *et al.*, 1996) and *S. pombe* (Patterson *et al.*, 1999), invokes the formation of a cruciform structure analogous to a Holliday structure (Holliday, 1964; Leach, 1994). After resolvase cuts across the cruciform in a precise manner (Figure 9), a hairpin can form after DNA ligase seals the gap between the 3' and 5' ends of the different DNA strands. A dicentric chromosome is also formed. If the large hairpin segregates with an intact chromosome I, a cell resistant to LiCl results. As in the model shown in Figure 8A, one more round of replication is required to form the mature double-stranded amplicon and additional rounds of replication can generate multiple copies of the amplicon.

The sequence of the novel joint reveals that the junction formed (Figure 9) is consistent with the expected products of *S. pombe* resolvase, which cuts Holliday junctions across the axis of symmetry and prefers phosphodiester bonds 3' to thymidine residues, cutting 3' to the cruciform structure (Whitby and Dixon, 1997; Oram *et al.*, 1998). Resolvase cuts within 6 bp of the junction in most cases. The locations within the cruciform structure at which subsequent excision is required or not required for our model are indicated in Figure 9. The areas lacking symmetry are also indicated; the lack of symmetry prevents this cruciform from being a true Holliday structure. The sequence of the novel joint of the 225-kb amplicon suggests that the cut may have occurred either 2 or 4 bp away from the cruciform if mismatch repair has occurred. Additional experiments to study the effects of base pair changes in this region should reveal whether resolvase plays a key role in forming the amplicon. For example, the removal of thymidine residues from this region should prevent amplification if resolvase is required.

Previous work has shown that mutations in the DNA-checkpoint pathway increased the frequency of *sod2* amplification (Patterson *et al.*, 1999). We now find that strains defective for ligase or for both topoisomerase I and topo-



**Figure 9.** Possible sites for resolvase action on the cruciform structure. Only the base of the cruciform is shown. Triangles indicate predicted sites of resolvase action. Black triangles, sites of resolvase action that would require subsequent mismatch excision repair to yield observed amplicon. White triangles, sites that would not require such excision. Carrot shape, sites lacking symmetry.

isomerase II activity also have greatly increased frequencies of amplification. Both types of mutation increase the likelihood of DNA breakage. Topoisomerase is necessary to untangle chromosomes, removing supercoiling and helping to maintain chromatin organization (Uemura *et al.*, 1987). DNA ligase is needed to seal single-strand DNA breaks. Together, these results make it likely that DNA breaks facilitate gene amplification in *S. pombe*. DNA breaks are also known to increase the frequency of mitotic recombination (Sipiczki *et al.*, 1990), and a single-strand break has been invoked in our replication model. The genetic data are consistent with both models for generating the 225-kb amplicon and the recombination model for the 180-kb amplicon. The aberrant replication model (Figure 8A) requires a single-strand break, and the recombination-resolvase model (Figure 8B) does not require DNA breakage at all. However, mitotic recombination is increased by DNA breakage and also by mutations in DNA ligase (Sipiczki *et al.*, 1990). Analysis of amplification frequencies in strains deficient in mitotic recombination may help to rule out this class of mechanisms for generating the 225-kb amplicon (see Gysler-Junker *et al.*, 1991).

We have presented the novel joint sequences for two different *sod2* amplicon structures. In the first case, the sequence of the novel joint indicates that homologous recombination is responsible for generating the 180-kb amplicon. In the second case, the sequence of the novel joint indicates that inverted repeats play a key role in generating the 225-kb

amplicon. Two different mechanisms, one involving aberrant replication and the other involving resolvase action on a cruciform structure, were presented as models of the mechanism of amplification by means of inverted repeats. All three models presented for generating the amplicons are expected to be stimulated by the presence of DNA breaks, as observed in our genetic data. Further studies, both molecular and genetic, should elucidate the mechanistic details of how the 225-kb palindromic *sod2* amplicon is formed. We have made observations consistent with circular amplicons being formed in this system. In both wild-type and mutant strains, megabase-size extrachromosomal amplicons have been observed. In one case, the amplicon was observed to migrate differently when different pulse times were used, consistent with it being circular. These observations have not been investigated further. However, they add to the argument that amplification in *S. pombe* may parallel amplification in higher organisms. The conservation observed between amplification in *S. pombe* and other organisms suggests that detailed studies in fission yeast will be relevant to the analysis of amplification mechanisms in higher eukaryotes.

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