Note

A 160-bp Palindrome Is a Rad50•Rad32-Dependent Mitotic Recombination Hotspot in *Schizosaccharomyces pombe*

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ABSTRACT

Palindromic sequences can form hairpin and cruciform structures that pose a threat to genome integrity. We found that a 160-bp palindrome (an inverted repeat of 80 bp) conferred a mitotic recombination hotspot relative to a control nonpalindromic sequence when inserted into the *ade6* gene of *Schizosaccharomyces pombe*. The hotspot activity of the palindrome, but not the basal level of recombination, was abolished by a *rad50* deletion, by a *rad50*S "separation of function" mutation, or by a *rad32-D25A* mutation in the nuclease domain of the Rad32 protein, an Mre11 homolog. We propose that upon extrusion of the palindrome the Rad50•Rad32 nuclease complex recognizes and cleaves the secondary structure thus formed and generates a recombinogenic break in the DNA.

DNA sequences that can adopt secondary structures can be unstable when present in the genome (Leach 1994). Mini-satellites such as CTG repeats that can adopt hairpin-like structures as well as palindromic sequences are unstable in the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and humans (Gordenin *et al.* 1993; Henderson and Petes 1993; Ruskin and Fink 1993; Sarkar *et al.* 1998; Richard and Pâques 2000; Bzymek and Lovett 2001; Edelmann *et al.* 2001). Instability of such structures can be deleterious, as observed in *E. coli* and humans (Leach 1994; Edelmann *et al.* 2001).

Depending on their size and their location in the genome, palindromic sequences display different degrees of stability and recombination stimulation. This behavior is thought to be dependent on their propensity to extrude and thereby form hairpin loops or cruciform structures. In *S. cerevisiae*, short palindromes (26 bp) appear not to extrude during vegetative growth and are infrequently repaired in heteroduplex DNA formed during meiotic recombination (Nag *et al.* 1989). Palindromes of 60–160 bp (hereafter called middle-sized palindromes, or M-pals) are frequently excised from the genome during mitotic growth (Gordenin *et al.* 1993; Henderson and Petes 1993; Ruskin and Fink 1993). This reaction depends on the presence of small (4–9 bp) direct repeats in the vicinity of the M-pals and on the replication machinery. A 140-bp M-pal is also a site of a DNA double-strand break (DSB) during meiosis in *S. cerevisiae* (Nag and Kurst 1997). Although longer palindromes (L-pals, or palindromes >600 bp) are mitotic recombination hotspots in *S. cerevisiae*, M-pals have not been reported to display such an activity (Gordenin *et al.* 1993; Lobachev *et al.* 1998, 2000; Nasar *et al.* 2000). L-pal-dependent recombination hotspot activity in *S. cerevisiae* likely stems from the propensity of these sequences to extrude into hairpins or cruciforms and from their subsequent cleavage or processing by the Rad50•Mre11•Xrs2 complex (Lobachev *et al.* 2002). M-pal mitotic instability as well as M-pal-dependent meiotic DSB formation in *S. cerevisiae* argue that these sequences do extrude during mitotic growth as well as during meiosis. These observations suggest that, in *S. cerevisiae*, an extruded M-pal either is not detected by the mitotic recombination machinery (including the Rad50•Mre11•Xrs2 complex) or is recognized and processed by a nonrecombinogenic pathway.

Since meiotic recombination displays important differences in *Schizosaccharomyces pombe* and in *S. cerevisiae* (Fox and Smith 1998; Young *et al.* 2002), we have compared the behavior of an M-pal in *S. pombe* with that reported in *S. cerevisiae*. We found, as in *S. cerevisiae*, that an M-pal conferred a meiotic recombination hotspot and led to meiotic DSB formation (J. A. Farah, W. W. Steiner and G. R. Smith, unpublished data). We report here that the M-pal was also a strong mitotic recombina-

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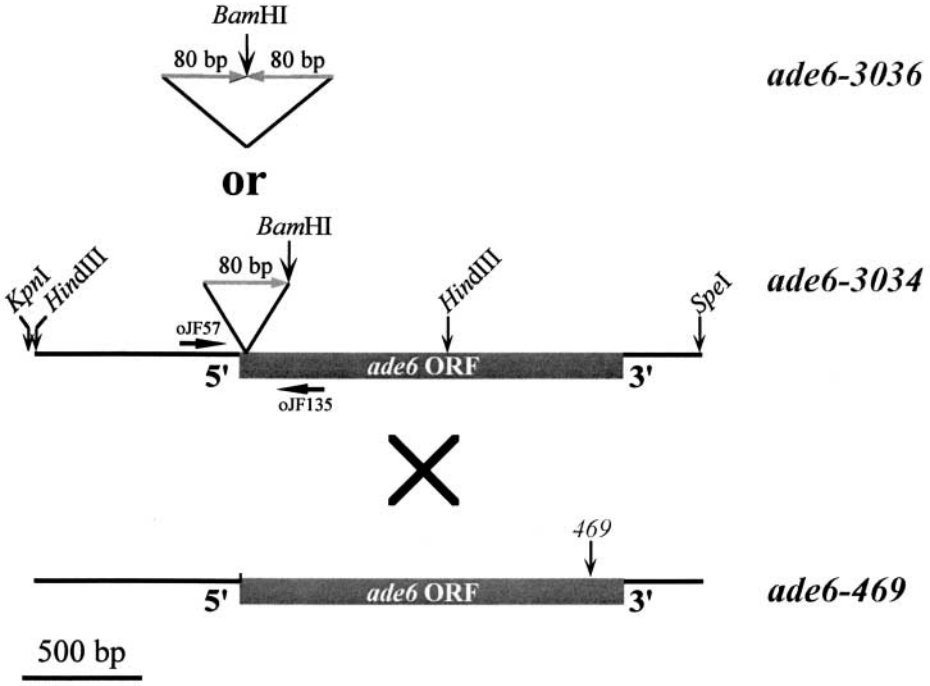

FIGURE 1.—*ade6* alleles used in this study. A 2859-bp *Pvu*II-*Spe*I fragment containing the *ade*$^+$ gene from pAS1 (SZANKASI *et al.* 1988) was cloned into the *Eco*RV-*Spe*I sites of pKS(+) (Stratagene, La Jolla, CA) to give plasmid pJF63. One or two copies of an 80-bp oligonucleotide were inserted at the unique *Bam*HI site of plasmid pJF63 to give plasmids pJF134 (*ade6-3034* control) and pJF136 (*ade6-3036* M-pal), respectively. The oligonucleotide corresponds to the *mat-a-stk* sequence from *S. cerevisiae* (positions 2044–2119 relative to GenBank sequence of the *MAT***a** locus; RAY *et al.* 1991). The inserted DNA is not drawn to scale. Both alleles are Ade$^-$. Primers oJF57 (5′ TGCTTGGAAAT GTAACGATGACAG 3′) and oJF135 (5′ TGAATGCATCGCAGAGTTGCA GGAG 3′) were used for PCR analysis. To transfer the *ade6-3034* and *ade6-3036* alleles to the chromosome, *Hin*dIII fragments of 1801 bp (from pJF134) or 1881 bp (from pJF136) were purified and used to transform strain GP2638 to Ade6$^-$ (red on limiting adenine EMM2 plates; FOX *et al.* 1997). To place the *ade6-3034* and *ade6-3036* alleles on an *S. pombe* replicative plasmid, *Spe*I-*Kpn*I fragments of 3067 bp (*ade6-3036* from plasmid pJF136) or 2987 bp (*ade6-3034* from plasmid pJF134) were cloned into the *Bam*HI-*Kpn*I sites of vector pFY20 (noncompatible ends were blunted with the Klenow enzyme; LI *et al.* 1997) to give plasmids pJF138 and pJF141, respectively. The *ade6-469* allele is a C-to-T transition that creates a stop codon 1445 bp downstream from the *Bam*HI M-pal insertion site (SZANKASI *et al.* 1988).

tion hotspot in *S. pombe* in contrast to *S. cerevisiae*. This hotspot was dependent on the Rad50·Rad32 complex, a putative structure-specific nuclease.

Mitotic recombination associated with a 160-bp M-pal was measured both in a chromosome-by-chromosome system in diploid strains and in a plasmid-by-chromosome system in haploid strains. The alleles used are shown in Figure 1. Briefly, the *ade6* alleles were constructed by inserting, at the unique *Bam*HI site of the *ade6* open reading frame, either one copy (the *ade6-3034* control allele) or two copies in opposite orientation (the *ade6-3036* M-pal allele) of an 80-bp oligonucleotide derived from the *MAT***a** locus of *S. cerevisiae*. These alleles were either integrated into the chromosomal *ade6* locus or present on an *S. pombe* replicative plasmid. For scoring *ade6*$^+$ recombinants these alleles were allowed to recombine with the *ade6-469* allele present either on the pade6-469 plasmid in haploids (SZANKASI *et al.* 1988) or on the homologous chromosome in diploids. Mitotic recombination rates were determined according to the method of the median (LEA and COULSON 1949).

We first determined mitotic recombination rates at *ade6* in diploid *rad*$^+$ strains. The *ade6*$^+$ recombination rate in a strain containing the M-pal was 56-fold higher than that observed in a control strain: 280 recombination events per 10^6 cell divisions compared to 5 recombination events per 10^6 cell divisions for strains GP3486 (*ade6-3036/ade6-469*) and GP3484 (*ade6-3034/ade6-469*), respectively (Tables 1 and 2). Similarly, the *ade6*$^+$ recombination rate in a haploid strain containing the M-pal on the chromosome was ~54-fold higher than that observed in a control strain: 700×10^{-6} compared to 13×10^{-6} for strains GP3019 (*ade6-3036* pade6-469) and GP3017 (*ade6-3034* pade6-469), respectively (Table 2). The latter recombination rate was comparable to the rate previously determined with equivalently spaced single-base-pair markers in *ade6* (~37×10^{-6}; PONTICELLI *et al.* 1988). A chromosomal *ade6* allele with two copies of the 80-bp fragment in a direct repeat orientation was also devoid of hotspot activity in the chromosome-by-plasmid recombination assay in a haploid strain (data not shown). Hence, a 160-bp M-pal in the *ade6* gene was a strong mitotic recombination hotspot in *S. pombe*.

We next tested whether the M-pal-dependent hotspot activity was observed when the M-pal was present on a multicopy plasmid in haploid strains. Plasmids pJF138 (*ade6-3036* M-pal) and pJF141 (*ade6-3034* control) were introduced into strain GP2947 (with the *ade6-469* allele on the chromosome; Table 1). Transformants with the control plasmid (pJF141) showed a recombination rate at *ade6* (~7×10^{-6}) that was 4- to 19-fold lower than that of transformants with the M-pal-containing plasmid (pJF138; Table 3). M-pal transformant T1 gave a value of 135×10^{-6}, while M-pal transformant T2 gave a recombination rate of 26×10^{-6}.

TABLE 1

***S. pombe* strains**

Strains	Genotype[a]
GP2638	*h*$^+$ *leu1-32 ura4-294*
GP2947	*h*$^+$ *ade6-469 leu1-32 ura4-294*
GP3017	*h*$^+$ *ade6-3034 leu1-32 ura4-294*
GP3019	*h*$^+$ *ade6-3036 leu1-32 ura4-294*
GP3125	*h*$^+$ *ade6-3034 leu1-32 ura4-294 rad50::kanMX6*[b]
GP3127	*h*$^+$ *ade6-3036 leu1-32 ura4-294 rad50::kanMX6*
GP3216	*h*$^+$ *mat1PΔ17::LEU2 ade6-3034 leu1-32 ura4-D18 his3-D1 rad51-D1::his3*$^+$[c]
GP3219	*h*$^+$ *ade6-3034 leu1-32 ura4-294 rad50S-K81I*[d]
GP3220	*h*$^+$ *ade6-3036 leu1-32 ura4-294 rad50S-K81I*
GP3259	*h*$^+$ *mat1PΔ17::LEU2 ade6-3036 leu1-32 ura4-D18 his3-D1 rad51-D1::his3*$^+$
GP3285	*h*$^+$ *mat1PΔ17::LEU2 ade6-3034 leu1-32 ura4-D18 rad32-D25A*[e]
GP3287	*h*$^+$ *mat1PΔ17::LEU2 ade6-3036 leu1-32 ura4-D18 rad32-D25A*
GP3484	*h*$^+$*/h*$^-$ (*smt-0*) *ade6-3034/ade6-469 ura4-294/ura4-294 leu1-32/leu1*$^+$ *his7*$^+$*/his7-366*
GP3486	*h*$^+$*/h*$^-$ (*smt-0*) *ade6-3036/ade6-469 ura4-294/ura4-294 leu1-32/leu1*$^+$ *his7*$^+$*/his7-366*
GP3600	*h*$^+$*/h*$^-$ (*smt-0*) *ade6-3034/ade6-469 ura4-294/ura4-294 leu1-32/leu1*$^+$ *his7*$^+$*/his7-366 rad50S-K81I/rad50S-K81I*
GP3601	*h*$^+$*/h*$^-$ (*smt-0*) *ade6-3036/ade6-469 ura4-294/ura4-294 leu1-32/leu1*$^+$ *his7*$^+$*/his7-366 rad50S-K81I/rad50S-K81I*

[a] Genealogies are available upon request.
[b] Hartsuiker *et al.* (2001).
[c] R. Kraehenbuehl and J. Kohli (personal communication).
[d] E. Hartsuiker (unpublished data).
[e] K. Mizuno and K. Ohta (unpublished data).

The nature of the difference between these two transformant types is not clear, but the higher-frequency T1 type is more common. Among 12 additional transformants, 11 behaved like T1 and one like T2. Upon extraction and analysis of plasmids from the T1-like and T2-like transformants, no restriction site or sequence differences could be detected between the two (data not shown). Transformants of strain GP2947 with the plasmids extracted from the T1-like and T2-like transformants showed *ade6* recombination frequencies similar to those of T1. Hence, the difference in the recombination rates between T1 and T2 is not a heritable property of the plasmid; it may stem from an epigenetic change in the plasmid or a genetic change in the host strain upon the initial transformation. Nevertheless, the plasmid-borne M-pal was a mitotic recombination hotspot when present on an extrachromosomal plasmid.

In summary, the results of Tables 2 and 3 clearly showed that, in an otherwise wild-type background, an M-pal was a mitotic recombination hotspot in *S. pombe* whether present on the chromosome or on a plasmid, although the hotspot activity was lower in the latter situation than in the former. These results suggest that the secondary structure adopted by the 160-bp M-pal is responsible for the observed hotspot activity at *ade6*.

One possibility is that the M-pal forms a hairpin structure that is recognized and cleaved by a nuclease, thus generating a recombinogenic lesion such as a DSB. In *E. coli*, palindrome-dependent inviability is dependent on the SbcCD complex (Leach 1994). This complex cleaves hairpin loops *in vitro* (Connelly *et al.* 1998). A related complex in eukaryotes, Rad50•Mre11•Xrs2 (Nbs1), is involved in DNA-damage repair and meiotic recombination (Johzuka and Ogawa 1995; Haber 1998). The human Rad50•Mre11•Nbs1 complex and the yeast Rad50•Mre11 complex are also nucleases that cleave hairpin DNA *in vitro* (Paull and Gellert 1999; Trujillo and Sung 2001). The overall architecture of these complexes involves the association of a structural-maintenance-of-chromosomes-type subunit (SbcC or Rad50) with a phosphoesterase enzyme (SbcD, Mre11, or Rad32, the *S. pombe* homolog; Tavassoli *et al.* 1995; Connelly *et al.* 1998; Hopfner *et al.* 2001).

The sequences of Mre11-related polypeptides from different species share four conserved esterase motifs; these motifs (I–IV) are important for nuclease activity *in vitro* (Furuse *et al.* 1998; Usui *et al.* 1998; Moreau *et al.* 1999). Regardless of the severity of their mitotic phenotypes, all reported esterase-motif mutants accumulate unprocessed DSBs during meiosis (Furuse *et al.* 1998; Usui *et al.* 1998; Moreau *et al.* 1999). This latter phenotype is reminiscent of that observed in the *rad50S-K81I* mutant of *S. cerevisiae* in which Lys-81 is changed to Ile (Alani *et al.* 1990; Cao *et al.* 1990). Recently, *S. pombe* strains with the corresponding *rad50-K81I* (hereafter *rad50*S) allele were also found to accumulate meiotic DSBs as in *S. cerevisiae* (Young *et al.* 2002). The *S. cerevisiae rad50S-K81I* allele was thought to have minimal defects during vegetative growth (Alani *et al.* 1990), but a recent report showed that when recombination is induced on an inverted repeat substrate, the *rad50*S allele favors break-induced replication over DSB repair (Rat-

TABLE 2

M-pal-dependent recombination hotspot activity and *rad* gene dependence in diploid and haploid strains

Strain	*ade6* chromosomal alleles	Genetic background	Recombination rate[a] (events per 10^6 divisions)		
			Experiment 1[b]	Experiment 2[c]	Mean[d]
Haploid[e]					
GP3017	*ade6-3034*	+	12 (10)	13 (7)	13
GP3019	*ade6-3036*	+	720 (10)	680 (7)	700
GP3219	*ade6-3034*	*rad50*S	10 (9)	ND	10
GP3220	*ade6-3036*	*rad50*S	19 (9)	ND	19
GP3125	*ade6-3034*	*rad50::kanMX6*	14 (9)	18 (5)	16
GP3127	*ade6-3036*	*rad50::kanMX6*	11 (9)	14 (5)	13
GP3285	*ade6-3034*	*rad32-D25A*	19 (9)	11 (4)	15
GP3287	*ade6-3036*	*rad32-D25A*	9 (9)	13 (4)	11
GP3216	*ade6-3034*	*rad51::his3*$^+$	3 (9)	2 (8)	3
GP3259	*ade6-3036*	*rad51::his3*$^+$	57 (9)	43 (8)	50
Diploid[f]					
GP3484	*ade6-3034*	+/+	3 (9)	6 (7)	5
GP3486	*ade6-3036*	+/+	270 (9)	290 (7)	280
GP3600	*ade6-3034*	*rad50*S/*rad50*S	7 (7)	9 (7)	8
GP3601	*ade6-3036*	*rad50*S/*rad50*S	11 (7)	10 (7)	11

ND, not done.

[a] Isolated colonies were inoculated into 5 ml of Difco nitrogen-base minimal liquid medium with the appropriate additives (for the haploid strains) or yeast extract liquid (for the diploid strains). The cultures were incubated for 2 or 3 days at 30°, at which point 1-ml aliquots were washed twice with water and appropriate dilutions plated on Difco yeast extract agar (YEA) for total viable cell counts and on YEA + guanine (80 μg/ml) for Ade$^+$ recombinant counts (Cummins and Mitchison 1967). Alternatively, cells were plated on nitrogen-base minimal agar (NBA) with appropriate additives for total viable cell counts and on NBA-adenine + guanine for Ade$^+$ recombinant counts. Plates were incubated at 32°. Typically, the total number of cells in the cultures ranged between 10^7 and 10^8, depending on the strain. Experiments 1 and 2 were done on different days. Recombination rates were measured according to the method of the median (Lea and Coulson 1949) with the number of cultures noted in parentheses; for even numbers of cultures, the median was the mean of the two medial values.

[b] The fluctuation tests were based on independent cultures of one transformant for each strain.

[c] Except for strains GP3017, GP3019, and the diploid strains, the fluctuation tests of experiment 2 were based on cultures of independent transformants for each strain.

[d] Mean of experiment 1 and experiment 2. When only one experiment was performed, the values obtained were reported in that column.

[e] The indicated haploid strain was transformed with plasmid pade6-469, which carries the *ade6-469* allele and the *S. cerevisiae URA3* gene as the selection marker (Szankasi *et al.* 1988; see Figure 1).

[f] The *ade6-469* allele was present on the second homolog.

tray *et al.* 2001). On the three-dimensional structure of the *Pyrococcus furiosus* Rad50 ATP-binding domain, the *rad50*S mutations cluster to a region of the protein that may interact with other proteins (Hopfner *et al.* 2000).

An attractive view is that the *S. pombe* Rad50•Rad32 complex is directly responsible for the cleavage of the hairpin formed by the extrusion of the M-pal. Although a complex between Rad50 and Rad32 has not been reported in *S. pombe*, we infer such a complex by analogy to the *S. cerevisiae* and human homologs. We first tested whether the M-pal-dependent mitotic recombination hotspot was dependent on the Rad50 protein in *S. pombe* and found it to be (Table 2). In the M-pal haploid strain GP3127 (*ade6-3036 rad50*Δ pade6-469) the *ade6*$^+$ recombination rate (13×10^{-6}) was very close to those of the control strains GP3017 (*ade6-3034 rad50*$^+$ pade6-469; 13×10^{-6}) and GP3125 (*ade6-3034 rad50*Δ pade6-469; 16×10^{-6}) with the nonpalindromic insertion at *ade6*. Hence, in the absence of the Rad50 protein, the hotspot activity of the M-pal was eliminated but the basal recombination rate was not greatly affected.

To test whether the M-pal-dependent hotspot was dependent on particular functions of the Rad50•Rad32 complex, we measured *ade6*$^+$ recombination rates in the presence of the non-null alleles *rad50*S and *rad32-D25A* (with an Asp-to-Ala change at the highly conserved position 25 in esterase motif I). The M-pal-dependent hotspot effect, but not the basal recombination level, was abrogated in these two mutant backgrounds. The M-pal haploid strains GP3220 (*ade6-3036 rad50*S pade6-469) and GP3287 (*ade6-3036 rad32-D25A* pade6-469)

TABLE 3

M-pal on a plasmid is a recombination hotspot

Transforming plasmid[a]	Recombination rate[b] (events per 10^6 divisions)
pJF141 (*ade6-3034*)	7
pJF138 (*ade6-3036*) T1[c]	135
pJF138 (*ade6-3036*) T2[c]	26

[a] The strain transformed was GP2947 (*ade6-469*).

[b] For each transformant, the recombination rate was based on nine independent cultures. See also footnote *a* to Table 2.

[c] T1 and T2 are two independent transformants of strain GP2947 with plasmid pJF138. In an independent experiment, among 12 transformants analyzed, 11 behaved as T1 and 1 behaved as T2.

showed *ade6*$^+$ recombination rates of 19×10^{-6} and 11×10^{-6}, respectively, which are not very different from the basal rates measured in the respective control strains GP3219 (*ade6-3034 rad50*S pade6-469; 10×10^{-6}) and GP3285 (*ade6-3034 rad32-D25A* pade6-469; 15×10^{-6}) with no M-pal. Similar results were also observed with diploid strains homozygous for the *rad50*S allele (Table 2). The M-pal-dependent recombination hotspot was eliminated in strain GP3601 (*ade6-3036/ade6-469 rad50*S/*rad50*S) with a recombination rate (11×10^{-6}) similar to that of the control strain GP3600 (*ade6-3034/ade6-469 rad50*S/*rad50*S; 8×10^{-6}).

Taken together, the above results suggest that a nuclease-proficient Rad50•Rad32 complex is necessary for the recombination hotspot activity of the M-pal inserted in the *ade6* gene of *S. pombe*. Although the *S. pombe* Rad32-D25A polypeptide was not tested directly for nuclease activity *in vitro*, the *S. cerevisiae* Mre11-D16A polypeptide (with the same amino-acid change at the homologous position as in Rad32-D25A) shows no *in vitro* nuclease activity despite wild-type affinity for DNA binding (Furuse *et al.* 1998).

If the recombination hotspot is due indeed to nuclease cleavage of the M-pal and DSB formation at that site, one prediction, according to two DSB repair models (Resnick 1976; Szostak *et al.* 1983), is that the the M-pal allele should be a recipient of wild-type information when recombining nonreciprocally with the *ade6-469* allele. Because the 102 *ade6*$^+$ recombinants analyzed from strains GP3484 and GP3486 (experiment 1 of Table 4) segregated red colonies upon sporulation (data not shown), we conclude that these recombinants were heterozygous diploids (*ade6*$^+$/*ade6*$^-$). Since the majority of these had lost the insertion (see below), it is reasonable to assume that *ade6*$^+$ recombinants derive from nonreciprocal recombination (gene conversion). We determined the frequency of conversion of the *ade6-3036* (M-pal) and the *ade6-3034* (control) alleles in *rad*$^+$ diploid strains (Table 4). In strain GP3486 (*ade6-3036/ade6-469*), the M-pal allele was converted to wild type with a frequency of ~98%, significantly higher than the conversion of the *ade6-3034* control allele in strain GP3484 (*ade6-3034/ade6-469*, 70%, contingency $\chi^2 = 28$, $P \ll 0.001$). The *ade6*$^+$ conversion frequency in strain GP3484 (*ade6-3034/ade6-469*, 70%) was higher than 50%, the expected value if there were no bias for conversion between the two recombining alleles. One explanation for this bias could be due to the nature of the *ade6-3034* allele, an insertion, that could be recognized and eliminated more efficiently than a point mutation in heteroduplex DNA by the mismatch repair or the nucleotide-excision repair machinery of the cell. Hence, the M-pal had a tendency to favor its own conversion to wild type as predicted.

A second prediction is that in a *rad* mutant that abolishes the hotspot activity of the M-pal, the conversion frequency of both the M-pal and the control allele should be similar, with no preference for either being converted to wild type. This was indeed the case when conversion frequencies were determined in the diploid strains homozygous for *rad50*S (Table 4). Strain GP3601 (*ade6-3036/ade6-469 rad50*S/*rad50*S) converted the M-pal

TABLE 4

Inheritance of *ade*$^+$ information in diploid strains

Diploid strain	*ade6* chromosomal alleles[a]	Genetic background	Conversion of *ade6-3034* and *ade6-3036* (% of total Ade$^+$ tested)		
			Experiment 1	Experiment 2	Mean[b]
GP3484	*ade6-3034*	+/+	34/51[c] (67)	36/50 (72)	70
GP3486	*ade6-3036*	+/+	50/51 (98)	49/50 (98)	98
GP3600	*ade6-3034*	*rad50*S/*rad50*S	29/50 (58)	24/51 (47)	53
GP3601	*ade6-3036*	*rad50*S/*rad50*S	31/50 (62)	36/51 (71)	67

[a] The *ade6-469* allele was present on the second homolog.

[b] Mean percentage from experiment 1 and experiment 2.

[c] Number of convertants among the number of Ade$^+$ recombinants analyzed. Conversion of the *ade-3034* and *ade6-3036* alleles was determined by PCR amplification of DNA from Ade$^+$ colonies using primers oJF57 and oJF135 (Figure 1). The PCR fragments from the mutant alleles were distinguished from each other and from the wild-type fragment by electrophoresis on a 1.5% agarose gel.

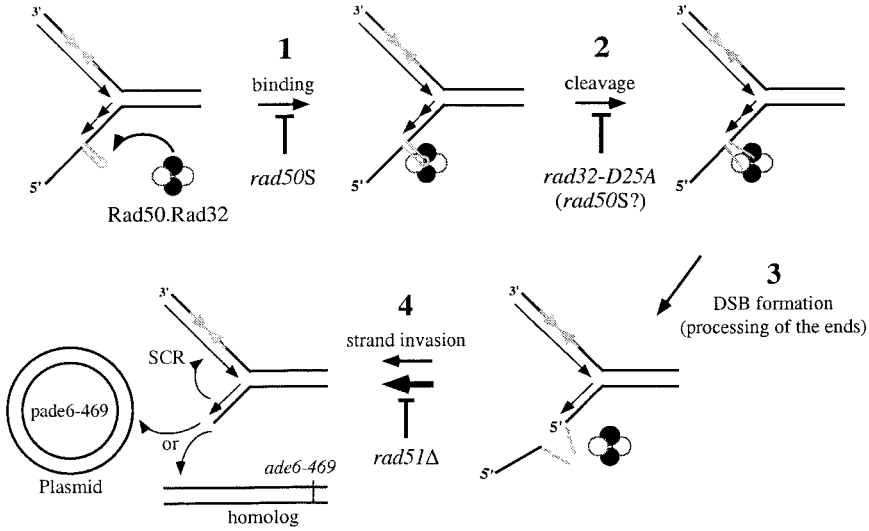

FIGURE 2.—Model for the M-pal-induced recombination hotspot activity. During S phase the M-pal extrudes on the lagging DNA strand (LEACH 1994). The Rad50•Rad32 complex recognizes (step 1) and binds to the secondary structure thus generated. The hairpin is cleaved (step 2) and processed (step 3) by the endonuclease activity of the Rad32 subunit, generating a DSB that can invade and recombine (step 4) with the replicated sister chromatid (sister chromatid recombination, SCR) or, when available, recombine with a homologous sequence on a plasmid or on a homolog. In the *rad50*S background, the Rad50S•Rad32 complex is unable to bind to or cut the hairpin (block of step 1 or 2) and hence no DSB is generated. In the *rad32-D25A* background, the Rad50•Rad32-D25A complex binds to the extruded M-pal, but no cleavage or processing ensues (block of steps 2 and 3). In the *rad51* deletion background, the major pathway for strand exchange (step 4, thick arrow) is abrogated, but minor *rad51*-independent pathways (thin arrow) allow lower efficiency recombination. In blocking steps 1 or 2 lagging strand DNA synthesis is expected to be halted at the secondary structure and could resume either when the hairpin unfolds or when the replication machinery "slips" past it. In the latter case, the M-pal is expected to be deleted from the genome (but see text).

67% of the time, a frequency similar to that of the control allele in strain GP3600 (*ade6-3034/ade6-469 rad50*S/*rad50*S, 53%, contingency $\chi^2 = 3.5$, $0.05 < P < 0.1$). Hence, in the absence of hotspot activity the M-pal allele was not converted preferentially to wild type. These results strongly suggest that the Rad50•Rad32 complex recognizes and cleaves the extruded M-pal. The DSB ends thus formed are subsequently processed (by trimming the nonhomologous extremities) and recombined with a homologous sequence with concomitant loss (conversion) of the M-pal insertion. In the *rad50*S mutant, the Rad50S•Rad32 complex cannot cleave the extruded M-pal, thus eliminating both the hotspot activity and the preferential conversion of that allele.

The involvement of the nuclease activity of Mre11 in mitotic DNA repair and recombination has been questioned on the basis of results obtained with certain *S. cerevisiae* esterase motif mutants. Some of these mutants with an Mre11 polypeptide devoid of detectable nuclease activity *in vitro* have no defect in the mating-type conversion reaction and are significantly more resistant to ionizing radiation than *mre11*Δ strains (MOREAU *et al.* 1999). However, we favor a direct role of the nuclease activity of the Rad50•Rad32 complex in the M-pal-dependent hotspot effect. The nuclease of the Rad50•Rad32 complex might be active on DNA substrates with secondary structures such as palindromes or microsatellites that might be rare in the genome but could form accidentally upon replication slippage or illegitimate recombination (MOORE *et al.* 1999; RICHARD and PÂQUES 2000). Perhaps such sequences are processed by the nuclease activity of the Rad50•Rad32 complex in an attempt to overcome their deleterious effects (RICHARD *et al.* 2000). In our system, where an artificial M-pal was introduced into the cell, this processing would result in the formation of a DSB at the M-pal and the recombination hotspot effect. Hence, one function of the Rad50•Rad32 complex could be to protect the genome from sequences that can form secondary structures known to cause genome instability.

The hotspot observed above could, however, be due to a less direct action of the Rad50•Rad32 complex on the M-pal. For instance, recombinogenic lesions could arise by a Rad50•Rad32-independent mechanism at the same rate on M-pal-containing and nonpalindromic alleles, but the subsequent processing of the lesion could favor recombination only with the M-pal-containing allele, thereby giving a higher recombination rate at *ade6*. In this case, it is reasonable to assume that the hotspot activity of the M-pal would be dependent on gene products acting at steps subsequent to the initial lesion. We therefore determined whether the hotspot effect of the M-pal depended on the *rad51*$^+$ gene product (also called *rhp51*$^+$; Table 2). The Rad51 protein is an *S. pombe* homolog of the *S. cerevisiae* Rad51 protein involved in DNA pairing and strand exchange between recombining DNA molecules, a step subsequent to the initial lesion (MURIS *et al.* 1993; SUNG 1994). The recombination rate in haploid strain GP3259 (*ade6-3036 rad51*Δ pade6-469; 50×10^{-6}) was 14-fold lower than that in strain GP3019 (*ade6-3036 rad51*$^+$ pade6-469; 700×10^{-6}; Table 2) but still significantly higher than that in strain GP3216 (*ade6-3034 rad51*Δ pade6-469) with the nonpalindromic substrate, 3×10^{-6}, near the limit of reliability. Hence, despite the dramatic decrease in the *ade6* recombination rates in the *rad51* deletion strains, an M-pal-dependent hotspot activity of at least 17-fold was still

present in this genetic background. These results reinforce the notion that the Rad50•Rad32 complex acts directly on the secondary structure of the M-pal, perhaps by generating a lesion that is subsequently processed to a DSB.

In the model in Figure 2, opening of the DNA helix during DNA replication allows extrusion of the M-pal on the less processively synthesized lagging strand (Trinh and Sinden 1991). Such a structure, which could stall the replication machinery and lead to breakage of the replication fork, could be processed by structure-specific nucleases (Leach 1994). The Rad50•Rad32 complex may accomplish that task by first binding (step 1) and then cleaving (steps 2 and 3) the secondary structure. A DSB that is repaired by recombination with a sister chromatid with retention of the M-pal ensues, as has been inferred in *E. coli* (step 4; Leach *et al.* 1997). Alternatively, the DSB can be repaired by recombining at high rate with a homologous plasmid or chromosome, thus displaying the hotspot activity described above. In the *rad50*S background, we propose that the Rad50•Rad32 complex is not properly targeted or bound to the hairpin or is not active on it (block at step 1). Because *rad50*S cells show near normal vegetative growth in contrast to *rad50* deletion strains (Hartsuiker *et al.* 2001; E. Hartsuiker, unpublished observations), the Rad50S•Rad32 complex appears to fulfill most of its other tasks in the cell. Only when special DNA features such as M-pals or special recombination substrates are present in the genome does a *rad50*S strain display a noticeable phenotype during vegetative growth (this work and Rattray *et al.* 2001). Alternatively, the Rad50 protein might control the nuclease activity of Rad32, and in the *rad50*S background a partial deficiency in that control might inhibit the activation of the nuclease at the M-pal, thus abrogating the hotspot. In the *rad32-D25A* background, the Rad50•Rad32 complex may bind to the hairpin but be unable to cleave or process it (block at step 2), thereby leaving this structure intact. Finally, in the *rad51* deletion background, the main pathway for DNA pairing and strand exchange is abolished (step 4), but minor Rad51-independent pathways still allow some recombination to occur without affecting the hotspot activity that is dependent on earlier events (steps 2 and 3).

An additional issue is the stability of M-pals in *S. pombe*. In the budding yeast *S. cerevisiae*, M-pals are unstable during mitotic growth and are excised from a plasmid or from the chromosome at a high rate (Henderson and Petes 1993; Ruskin and Fink 1993). The excision rate is increased in the presence of temperature-sensitive alleles of *POL1* (encoding Polα; Ruskin and Fink 1993) or *POL3* (encoding Polδ) at the semirestrictive temperature (Gordenin *et al.* 1993), suggesting that M-pal excision is intimately linked to replication on the lagging strand (Morrison *et al.* 1990).

To determine M-pal excision in *S. pombe*, we measured *ade6*$^+$ reversion rates. Strains GP3017 (*ade6-3034*) and GP3019 (*ade6-3036*), with no plasmid present, were plated on Ade$^+$ selective plates. For both strains, the *ade6*$^+$ reversion rate was $<1.3 \times 10^{-8}$ (95% confidence limit). Hence, the M-pal seemed stable when present in the chromosome, although 4-bp direct repeats flanked the M-pal, and DNA polymerase slippage at these repeats was expected to restore a wild-type *ade6*$^+$ sequence (Ruskin and Fink 1993). These 4-bp repeats might not be long enough, however, to allow polymerase slippage.

Although some biological processes are conserved between budding yeast and fission yeast, it is becoming increasingly clear that others are regulated differently despite conservation of the proteins involved (Forsburg 1999). The behavior of the M-pal may be an example of this difference: although the 160-bp M-pal is a meiotic hotspot and a site of meiotic DSB (J. A. Farah, W. W. Steiner and G. R. Smith, unpublished data), as expected from work in *S. cerevisiae*, a mitotic recombination hotspot at an M-pal has not been reported in the budding yeast.

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