Replication Protein A Is Required for Meiotic Recombination in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae*, meiotic recombination is initiated by transient DNA double-stranded breaks (DSBs). These DSBs undergo a 5′ → 3′ resection to produce 3′ single-stranded DNA ends that serve to channel DSBs into the RAD52 recombinational repair pathway. In *vitro* studies strongly suggest that several proteins of this pathway—Rad51, Rad52, Rad54, Rad55, Rad57, and replication protein A (RPA)—play a role in the strand exchange reaction. Here, we report a study of the meiotic phenotypes conferred by two missense mutations affecting the largest subunit of RPA, which are localized in the protein interaction domain (rfa1-t11) and in the DNA-binding domain (rfa1-t48). We find that both mutant diploids exhibit reduced sporulation efficiency, very poor spore viability, and a 10- to 100-fold decrease in meiotic recombination. Physical analyses indicate that both mutants form normal levels of meiosis-specific DSBs and that the broken ends are processed into 3′-OH single-stranded tails, indicating that the RPA complex present in these rfa1 mutants is functional in the initial steps of meiotic recombination. However, the 5′ ends of the broken fragments undergo extensive resection, similar to what is observed in rad51, rad52, rad55, and rad57 mutants, indicating that these RPA mutants are defective in the repair of the Spo11-dependent DSBs that initiate homologous recombination during meiosis.

**R**eplication protein A (RPA) is the eukaryotic counterpart of the *Escherichia coli* single-stranded DNA-binding protein (SSB), which was initially identified as an essential factor for replication *in vitro* (for a review, see Wold 1997) and later shown by *in vivo* and *in vitro* approaches to be required for most aspects of eukaryotic DNA metabolism: specifically, RPA is required in nucleotide excision repair (Coverley et al. 1991; Guzder et al. 1995), telomere maintenance (Smith et al. 2000), and homologous recombination (Sung 1994; Smith and Rothstein 1999; for a review, see Wold 1997). RPA is also a component of the surveillance mechanisms that link the recognition of defects in DNA metabolism with cell cycle progression (Brush et al. 1996; Cheng et al. 1996; Lee et al. 1998; Kim and Brill 2001; Pelliccioni et al. 2001).

In *Saccharomyces cerevisiae* as in other eukaryotes, RPA is composed of three subunits of 69, 36, and 13 kD that are encoded by the three essential genes RFA1, RFA2, and RFA3, respectively (Heyer et al. 1990; Brill and Stillman 1991). The Rfa1 subunit bears the major single-stranded DNA (ssDNA)-binding activity (Brill and Stillman 1989; Heyer et al. 1990; Philipova et al. 1996; Brill and Bastin-Shanower 1998). Biochemical studies have delineated three distinct domains of the protein: the N-terminal domain [amino acids (aa) 1–170], which is involved in interactions of RPA with other proteins, including Pol α (Kim et al. 1996) and Rfc4 (Kim and Brill 2001); two central DNA-binding subdomains, A and B (aa 180–416); and a C-terminal domain (aa 450–616), which includes a third DNA-binding subdomain, C (Brill and Bastin-Shanower 1998) and is required for binding the Rfa2 and Rfa3 subunits (for a review, see Wold 1997). Several genetic screens have led to the identification of rfa1 mutations that confer different phenotypes. Some mutants are defective in intrachromosomal recombination (Longhese et al. 1994) and in the recombinational repair of induced double-stranded breaks (DSBs; Firmenich et al. 1995; Umezu et al. 1998), whereas others exhibit a stimulation of recombination among direct repeats (Smith and Rothstein 1995, 1999; Smith et al. 2000), UV irradiation and methyl methanesulfonate (MMS) sensitivities, growth thermosensitivity, and replication defects (Umezu et al. 1998; Kim and Brill 2001), or mutator phenotypes (Chen et al. 1998). Here, we have genetically and physically studied the phenotypes of two rfa1 mutants and the role of RPA in meiotic homologous recombination.

In *E. coli*, the RecA protein catalyzes the strand exchange reaction between two homologous DNA molecules. In eukaryotic cells, several structural homologs of bacterial RecA have been described: Rad51 (Aboussekha et al. 1992; Basile et al. 1992; Shinozuka et al. 1992; Benson et al. 1994), Rad55 (Lovett 1994), Rad57...
Rad54 (only in a chromosome. In vivo proteins to promote strand invasion of a homologous key intermediate is the substrate used by recombination dent DSBs that initiate homologous recombination. Bishop 1991; the production of 3 \text{H}11032 that could compete with Rad51 for binding on ssDNA.

Bergerat dependent DSBs (ton 1995; ssDNA nucleoprotein assembly in the presence of RPA Bergerat et al. 1997a) and their capacity to interact with Rad51 (RAD57 ssDNA-binding properties (Bergerat et al. 1998; and for a review, see Sung et al. 2000). The role of Rad52 and Rad55/Rad57 as cofactors for the Rad51 recombinase activity is likely related to their ssDNA-binding properties (Mortensen et al. 1996; Sung 1997a) and their capacity to interact with Rad51 (Shinohara et al. 1992; Hays et al. 1995; Johnson and Symington 1995; Shen et al. 1996), which may facilitate Rad51-ssDNA nucleoprotein assembly in the presence of RPA that could compete with Rad51 for binding on ssDNA. Interestingly, immunostaining of budding yeast meiotic cells showed that DSB-dependent Rad51 focus formation requires the presence of the Rad52, Rad55, and Rad57 proteins and that Rad52 and RPA extensively colocalize (Gasior et al. 1998).

During meiotic recombination, all of the genes of the RAD52 pathway (RAD52, RAD50, RAD51, RAD55, RAD57, MRE11, and XRS2) have essential but distinct roles (for a review, see Paques and Haber 1999). In S. cerevisiae, meiotic recombination is initiated by Spo11-dependent DSBs (Bergerat et al. 1997; Keeney et al. 1997), which form on one of a pair of homologous duplexes (Sun et al. 1989; Cao et al. 1990; Figure 1). The broken ends then undergo 5’ to 3’ processing leading to the production of 3’ single-stranded tails (Sun et al. 1991; Bishop et al. 1992; Vedel and Nicolas 1999). This key intermediate is the substrate used by recombination proteins to promote strand invasion of a homologous chromosome. In vivo, Rad51, Rad52, Rad55, Rad57, Rad54 (only in a rdh54 background), and Dmc1 are all required for the formation of joint molecules (Schwacha and Kleckner 1997; Shinohara et al. 1997). In the corresponding deletion mutant strains, unpaired DSBs accumulate in a hyperresected form (Bishop et al. 1992; Shinohara et al. 1992, 1997; Dresser et al. 1997; and this study).

The aim of the present study was to examine the effect of two RPA mutations (rfa1-t11 and rfa1-t48) on the formation and/or stabilization of the 3’ single-stranded DSB tail. These mutants are UV and MMS sensitive and deficient in DSB-induced homologous recombination in mitotic cells, as shown by their sensitivity to HO endonuclease-induced DSBs and defects in mating-type switching or in single-stranded-annelling recombina-

![Diagram of meiotic recombination](image)

**Figure 1.**—Initial steps of meiotic recombination (as originally proposed by Szostak et al. 1983) and the main proteins involved.

**MATERIALS AND METHODS**

**Plasmids and oligonucleotides:** A SalI-BamHI fragment from the plasmid pKU2-rfa1-t11, which bears the rfa1-t11 allele, was cloned into the same sites of pRS406 (Umezu et al. 1998), a URA3 vector (Sikorski and Hieter 1989). The plasmid pRS(t48) was constructed by introduction of the pKU1-rfa1-t48 SalI-BamHI fragment bearing the rfa1-t48 allele (Umezu et al. 1998) into pRS306. The plasmid pSTL11 (a gift from S. Lovett) contains the RAD55 gene disrupted by insertion of a SalI-XbaI LEU2 fragment into the internal SalI site (Lovett and Mortimer 1987). The HindIII fragment from pSTL11 was used for transformation. The plasmid p51::LEU2 (a gift from F. Fabre) was created by cloning a BamHI fragment bearing a RAD51 disruption cassette into the BamHI site of the vector pTZ18. The RAD51 disruption was created by the insertion of a Hpol fragment of LEU2 into the RAD51 Stud-Nrul sites, and the BamHI fragment of p51::LEU2 was used
for transformation. The plasmid pAS263 (a gift from N. Kleckner) was derived from pAS264, which contains a rad57::TRP1 construct (Schwacha and Kleckner 1997), by insertion of the URA3 gene at the BglII site. The BamHI-SphI fragment of pAS263 was used for transformation. The EcoRV-BglII AR44 internal fragment was used as a probe in Southern blotting experiments. It was isolated from pNPS104, which was created by transformation with Eco I-linearized pRS-t48 into haploid strains

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>Source</th>
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<tr>
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<td>MATa arg4Δ2060 leu2-3,112 ura3-52 trp1-289 cyhr</td>
<td>Rocco et al. (1992)</td>
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<tr>
<td>MGD131-102A</td>
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</tr>
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</tr>
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<tr>
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<td>H. Debrauwere</td>
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<tr>
<td>ORD5402</td>
<td>MATa arg4-Erv rfa1-t48</td>
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*The haploid strains correspond to the primary transformant and are derived from the MGD131-2C and MGD131-102A haploid strains.

*The rad50S-KI81 mutation is marked in cis by an URA3 insertion (Alani et al. 1990).
The arg4-E (Sigma, St. Louis), Zymolyase 20T (ICN), and an automated chromosomal DNA was extracted, purified, and digested by Southern blot analysis. For both Rocco bearing the arg4-E strains were chosen because they undergo a higher level of DSB formation and meiotic recombination than do wild-type strains (ROCCO and NICOLAS 1996). The rad51, rad54, and rad57 strains were obtained by one-step transformation with the respective plasmids (as described above) into MGD131-2C (Table 1). The rad52:LEU2 haploid strain (constructed by H. Debrouwere) corresponds to an insertion of a fragment from the pRNB92 plasmid (D. Schild) in which a LEU2 fragment is inserted into the internal BgII site of the RAD52 gene. The dmc1::URA3 diploid strain corresponds to an insertion by pop-in pop-out of a fragment from the pRNB92 plasmid (BISHOP et al. 1992). All the constructions were verified by Southern blot analysis. For both rfa1 mutants, MMS and UV sensitivity tests for strain verification were performed on plates. Stationary liquid cultures in YPD medium were counted and diluted and drops of different dilutions were deposited on YPD and irradiated with a 254-nm UV lamp or deposited on YPD + MMS (0.015%). MMS and UV sensitivities were assessed after 3–4 days of incubation at 30°C by comparison with untreated strains.

Standard media and culture conditions were used (AUSUBEL et al. 1987). Conditions for presporulation and sporulation were as previously described (RENSNICK et al. 1983; DE MASSY and NICOLAS 1993). MMS plates were prepared by the addition of MMS to YPD medium to a final concentration of 0.015%; the plates were kept at 4°C and used within 2 days after preparation. The entry into and progression through meiosis were assessed after 3–4 days of incubation at 30°C by comparison with untreated strains.

Detection of recombination frequencies: Tetrad dissection and random spore analyses were performed by standard methods at 30°C (AUSUBEL et al. 1987) using β-glucuronidase (Sigma, St. Louis), Zymolyase 20T (ICN), and an automated microdissector (Singer Instruments MSM). Tests for determination of the mating type or the presence of the specific ARG4 alleles were performed as described using appropriate tester strains (NICOLAS et al. 1989). The return-to-growth assays were performed as described (SHERMAN and ROMAN 1963). Cells were induced to sporulate and at different times aliquots were counted, diluted, and plated onto YPD plates to test survival and onto selective medium plates to monitor the appearance of Arg" prototrophs. The frequency of Arg" recombinants was calculated as the ratio of Arg" colonies to total colonies on YPD, for each time point. Meiotic recombination was also examined physically by Southern blot analysis of DNA extracted from diploids heteroallelic for the arg4-Erv mutants, MMS tails are provided in the legends to Figure 2A and Figure 3B.

Detection of meiotic DSBs: Chromosomal DNA was extracted from meiotic cells as described (ROCCO et al. 1992), digested with appropriate enzymes, and fractionated by electrophoresis through 0.7% agarose gels. The DNA fragments were then transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK) as described previously (ROCCO et al. 1992), with the exception that the DNA was fixed to the membrane by treatment with 0.4 N NaOH for 15 min and then rinsed in 0.5 M NaHPO₄, pH 7.3. The resulting membrane was prehybridized and hybridized for 24 hr according to CHURCH and GILBERT (1984). Labeling of the DNA probes was done by random priming according to the specifications of the manufacturer (ReadyPrime kit, Pharmacia, Piscataway, NJ) using 50 μCi of 3,000 Ci/mM [α-³²P]dCTP (Amersham). The membrane was then exposed to a phosphor screen and quantified with a phosphorimager system using ImageQuant software analysis (Storm, Molecular Dynamics, Sunnyvale, CA).

Detection of single-stranded DNA intermediates: Meiotic chromosomal DNA was extracted, purified, and digested ex-
**Figure 3.**—(A) Meiotic recombination defects of rfa1 mutants. Commitment to meiotic recombination in RFA1 (ORD2130), rfa1-t11 (ORD3256), and rfa1-t48 (ORD5402) strains was followed throughout sporulation by a return-to-growth assay. After transfer to sporulation medium, aliquots of cells were taken at different times, diluted, and plated onto YPD plates and on plates lacking arginine to monitor the appearance of Arg+ prototrophs. The frequency of recombination is calculated as the ratio of the number of Arg+ colonies to the total number of colonies on YPD plates (see also Table 2). (B) Physical detection of recombinant molecules at the ARG4 locus. Genomic DNA extracted from RFA1 (ORD2130), rfa1-t11 (ORD3256), and rfa1-t48 (ORD5402) meiotic cells at the indicated times was digested with EcoRV and BglII and subjected to Southern blot analysis using as a probe an EcoRV-BglII fragment (1016 bp) internal to ARG4. The positions of the parental (P1) and recombinant (R1) bands are indicated.

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**RESULTS**

**Reduced sporulation and spore viability in diploids homozygous for the rfa1-t11 and rfa1-t48 mutations:** To study the meiotic phenotypes conferred by the rfa1-t11 and rfa1-t48 mutations (Umez et al. 1998), we constructed diploids homozygous for these mutations (ORD3256 and ORD5402, respectively) by mating haploids containing the mutations of interest. These strains are derivatives of our standard haploid strains MGD131-2C and MGD131-102A (S288C background; see Rocco et al. 1992). The genotypes of these and all other strains used in this study are indicated in Table 1. For the sake of simplicity, the homozygous diploids will be referred to hereafter as RFA1, rfa1-t11, and rfa1-t48. In this strain background, we observe that ~60% of RFA1 diploid cells (ORD2130) sporulate after 2 days in sporulation medium (producing mainly four-spore tetrads), but that the rfa1-t11 diploid has a reduced sporulation efficiency (25%, corresponding to a 2- to 3-fold decrease relative to the RFA1 strain) while only 3% of rfa1-t48 diploid cells form tetrads, representing a 20-fold decrease (Table 2). To examine meiotic progression, we stained cells with DAPI at different times during sporulation and counted the number of cells with one, two, or four nuclei to determine the percentage of cells that had undergone the first or the second meiotic division. The results show that RFA1 and mutant (rfa1-t11 and rfa1-t48) cells exhibit an increase in the number of binucleated cells at 11 hr after transfer to sporulation medium and an increase in the percentage of tetranucleated cells at 24 hr, with maximal frequencies of ~60% for the RFA1 strain, 30% for the rfa1-t11 strain, and 12% for the rfa1-t48 strain. These results parallel the efficiency of sporulation as ascertained by light microscopy (Table 2). To test whether the sporulation defects of these mutants are accompanied by changes in spore viability, we dissected tetrads derived from each diploid. Up to 95% of RFA1 spores are viable and in all RFA1 tetrads three or four spores germinated and formed colonies. In contrast, we observed a severe reduction in spore viability for the rfa1-t11 (18.7% viable spores among 142 four-spore tetrads dissected) and the rfa1-t48 (7% viable spores among 84 four-spore tetrads) diploids (Table 2).

For these mutants, in most tetrads either no spores or only a single germinating spore germinated, indicating that each meiotic cell is affected by the rfa1 mutations. Microscopic examination of the dissection plates indicated that most of the spores did not germinate (nearly 90% for each mutant strain). The remaining spores that did not form visible colonies germinated but formed multiply budded structures with few cells, similar to what was previously described for rfa1 null alleles (Heyer et al. 1990). We conclude, therefore, that rfa1-t11 and rfa1-t48 homozygous diploids are defective in progressing through meiosis and in sporulation and that they generally give rise to inviable spores.

**Meiotic gene conversion is severely reduced in the rfa1-t11 and rfa1-t48 mutants:** We next examined the proficiency of the rfa1-t11 and rfa1-t48 diploids for meiotic recombination by genetic and physical means. The RFA1 (ORD2130), rfa1-t11 (ORD3256), and rfa1-t48
TABLE 2

Meiotic phenotypes of the rfa1 mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sporulation phenotypes</th>
<th>Recombination (Arg(^+) frequency)</th>
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<tbody>
<tr>
<td></td>
<td>Random spore analysis</td>
<td>Return-to-growth assay</td>
</tr>
<tr>
<td></td>
<td>efficiency(^a)</td>
<td></td>
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<tr>
<td></td>
<td>Spore viability(^b)</td>
<td></td>
</tr>
<tr>
<td>RFA1</td>
<td>5.5 × 10(^{-2}) (1)</td>
<td>1.0 × 10(^{-1}) (1)</td>
</tr>
<tr>
<td>rfa1-t11</td>
<td>8.0 × 10(^{-3}) (0.14)</td>
<td>1.4 × 10(^{-2}) (0.14)</td>
</tr>
<tr>
<td>rfa1-t48</td>
<td>2.4 × 10(^{-3}) (0.043)</td>
<td>1.1 × 10(^{-3}) (0.011)</td>
</tr>
</tbody>
</table>

All the data are mean values of at least three experiments.

\(^a\) Percentage of three- and four-spore tetrads among all cells.

\(^b\) Values within parentheses correspond to the ratio mutant over wild type (RFA1).

(ORD5402) diploids are heteroallelic for the arg4-Erv and arg4-Ebg mutations at the ARG4 hot spot of meiotic recombination (Nicolas et al. 1989; Rocco et al. 1992; de Massy and Nicolas 1993), allowing us to determine the frequency of recombination by random spore analysis. In the RFA1 strain, we observed a high frequency of Arg\(^+\) prototrophs, up to 5.5% (±1.5 × 10\(^{-3}\)) of all spores. These recombinants are due mostly to gene conversion events (Nicolas et al. 1989). In contrast, the frequency of Arg\(^+\) prototrophs was reduced by 7- and 23-fold, relative to the RFA1 diploid, among the progeny of the rfa1-t11 (8.0 ± 1.5 × 10\(^{-3}\)) and rfa1-t48 (2.4 ± 0.2 × 10\(^{-3}\)) diploids, respectively (Table 2). Because of the poor spore viability of rfa1 spores, which might make it impossible to accurately determine the frequency of cells that undergo recombination at the ARG4 locus, we also performed a return-to-growth analysis. This assay allows for the recovery of cells in which meiotic recombination is initiated but not completed (Sherman and Roman 1963; for details see materials and methods).

As shown in Figure 3A, in the RFA1 strain we observed a nearly 10,000-fold increase in the frequency of meiotic Arg\(^+\) recombinants, reaching 1.0 ± 0.2 × 10\(^{-1}\) 8 hr after transfer of the cells to sporulation medium. For the rfa1-t11 and rfa1-t48 diploids, we also observed an increase of the frequency of the Arg\(^+\) prototrophs over the mitotic frequency but the maximal frequency is decreased by 10- (1.4 ± 0.3 × 10\(^{-3}\)) and 100-fold (1.1 ± 1.0 × 10\(^{-3}\)), respectively, relative to RFA1 diploid (Figure 3A and Table 2). This large decrease of the meiotic recombination frequency in the mutant strains does not correlate with a loss of cell viability over the course of the return-to-growth experiment (at 24 hr, the cell viability is 100% for the RFA1 strain, 90% for the rfa1-t11 strain, and 80% for the rfa1-t48 strain).

Finally, we examined the formation of recombinant molecules during meiosis at the ARG4 locus by physical analysis. We monitored the appearance of the recombinant 1016-bp EcoRV-BglII restriction fragment (R1) by Southern blot analysis of DNA extracted from diploids at various times after transfer to sporulation medium. In the RFA1, rfa1-t11, and rfa1-t48 diploids, we began to detect the recombinant fragment at 8 hr (Figure 3B). Quantification of the recombinant band at 24 hr indicates that it is reduced about fivefold in rfa1-t11 mutants and fivefold or greater in rfa1-t48 mutants, confirming that both mutants are defective for meiotic gene conversion as compared with wild-type strains. Although the rfa1-t11 and rfa1-t48 mutants can form recombinant products, albeit at a lower level, like the rad51 and rad52 deletion mutants (Bishop et al. 1992; Shinohara et al. 1992; Ogawa et al. 1993a) these events do not appear to be sufficient to ensure normal progression through meiosis, as manifested by the strong effects of the rfa1 mutations on sporulation and spore viability. Consistent with its slower growth rate in vegetatively growing cells, the rfa1-t48 diploid is more impaired than is the rfa1-t11 diploid in meiotic events.

Meiotic DSBs form but undergo extended resection in the rfa1-t11 and rfa1-t48 mutants: To determine at which step meiotic recombination might be defective in rfa1-t11 and rfa1-t48 mutants, we monitored the appearance of the DSBs that initiate recombination. In wild-type diploids (RAD50), meiotic DSBs are detected as transient DNA fragments of heterogeneous size that reflect their processing (Sun et al. 1989, 1991; Gao et al. 1990). We first examined meiotic DSB formation in the 5’ intergenic region of the ARG4 locus (Sun et al. 1989). As shown in Figure 4A, we performed a side-by-side comparison of DSB formation at the ARG4 locus in RFA1, rfa1-t11, and rfa1-t48 diploids. We found that meiotic DSBs form in the three diploids. In the MGD background, the meiotic DSBs are transiently detectable in the RFA1 diploid between 5 and 11 hr after transfer to sporulation medium. Similarly, DSBs can be detected in the rfa1-t11 strain. However, we note that DSB frag-
fragments appear to be of similar size in the RFA1 of the DSB smears suggests that there are subtle differences between the RFA1 and the rfa1-t48 mutants. (B) Meiotic DSBs at RFA1 rad50S (ORD2130), RFA1 ARG4 (ORD3257), and rfa1-t48 rad50S (ORD3257) strains. A meiotic time course of the RFA1 rad50S strain (ORD2410), run independently, is shown in the bottom part of the figure.

Figure 4.—Detection of meiotic DSBs at the ARG4 locus in rfa1 strains. Cells were taken at 0, 5, 8, 11, and 24 hr after transfer to sporulation medium and treated as described previously. Genomic DNA was extracted from meiotic cells, digested with PstI, electrophoresed, transferred, and probed with a EcoRI-BglII fragment internal to ARG4 (see Figure 2, A and B). Positions of the parental and ARG4 DSB fragments are indicated by horizontal arrows. (A) Meiotic DSBs at ARG4 in the RFA1 (ORD2130), rfa1-t48 (ORD5402), and rfa1-t48 (ORD5402) strains. (B) Meiotic DSBs at ARG4 in the RFA1 (ORD2130), rfa1-t411 (ORD3256), and rfa1-t411 rad50S (ORD3257) strains. A meiotic time course of the RFA1 rad50S strain (ORD2410), run independently, is shown in the bottom part of the figure.

ments are more heterogeneous in size in the rfa1-t411 and the rfa1-t448 mutants than in the RFA1 strain (see below). Similar results were observed for DSB formation at the CYS3 locus on chromosome I (data not shown). A close comparative examination of the characteristics of the DSB smears suggests that there are subtle differences in the range of fragment sizes: namely, the largest fragments appear to be of similar size in the RFA1 and rfa1-t411 diploids but greater in size than the largest fragments of the rfa1-t448 strain. Moreover, the range of fragment sizes, as indicated by the width of the smear, is most restricted in the RFA1 diploid (100–150 bp), intermediate in the rfa1-t448 diploid (200–250 bp), and broadest (300 bp) in the rfa1-t411 diploid (Figure 4A). Also, in both mutants, we noted that the overall level of DSB fragments only slightly decreases at late times and does not completely disappear (Figure 4, A and B), which correlates with the reduced production of Arg+ cells and recombinant molecules (Figure 3, A and B).

To more accurately quantify the extent of breakage in the rfa1 mutants, we also examined DSB formation in diploids homozygous for the rad50S mutation, which accumulate DSB fragments. In rad50S mutants, DSB ends are neither resected nor repaired (Alani et al. 1990) since the Spo11 transesterase remains covalently attached (Keeney et al. 1997). In the rfa1-t411 rad50S diploid, DSBs form and accumulate as a discrete band similar to what is seen for a RFA1 rad50S diploid (Figure 4B). Quantitatively, the amount of DSB formation at 11 hr, measured as the ratio of the intensity of the DSB band to total DNA (parental + all DSB signals), is 11.6% for the RFA1 rad50S strain and 12.8% for the rfa1-t411 rad50S (Rocco and Nicolas 1996; this study). Altogether, these results demonstrate that the rfa1-t411 and rfa1-t448 mutants are not defective in meiotic DSB formation, but are affected in their processing, and that there are subtle differences between the rfa1-t411 and rfa1-t448 diploids.

The fate of DSB ends is the same in RFA1, rfa1-t411, and rfa1-t448 diploids: Considering the ssDNA-binding properties of the RPA complex (Brill and Stillman 1989; Wold et al. 1989) and the above observation that the rfa1-t411 and rfa1-t448 diploids have an abnormal distribution of DSB fragments, we examined each of the two complementary DNA strands on both sides of the DSB in both RFA1 and mutant diploids. For this purpose, MluI-NruI-digested genomic DNA was resolved on alkaline denaturing gels and probed with a single-stranded probe specific to the YHR015 ORF, near the ARG4 locus (Figure 2, A and B). With this probe, we observed the 3’ end of the single-stranded DNA on the right side of the ARG4 DSB site in the RFA1 RAD50, rfa1-t411 RAD50, and rfa1-t448 RAD50 diploids as a discrete band of the same length as that found in rad50S diploids, indicating that this strand is not resected (Figure 5). Under the same experimental conditions, the 3’ single-stranded fragment on the left side of the ARG4 DSB site was similarly detected with a probe specific to ARG4 (data not shown). This unresected 3’ strand DSB fragment accumulates in the rfa1-t411 and rfa1-t448 mutants but not in the RFA1 diploid. Altogether these results indicate that the deficiencies of the rfa1-t411 and rfa1-t448 mutants in DSB repair are not due to a defect in the formation or maintenance of the 3’ single-stranded
Comparisons of meiotic DSB formation in *rfa1-t11*, *rfa1-t48*, *rad51*, *rad52*, *rad55*, and *rad57* diploids: As previously reported for the *HIS4-LEU2* locus (data not shown), we wished to compare DSB formation, processing, and repair in *rfa1-t11* and *rfa1-t48* diploids and in *rad51*, *rad52*, *rad55*, and *rad57* diploids, using mutant strains obtained in the MGD background (Materials and Methods and Table 1). For strains with the *rad50S* mutation, we observed that the *RFA1*, *rad51*, *rad52*, *rad55*, and *rad57* diploids form and accumulate meiotic DSBs at a similar level, at both the *ARG4* (Figure 6B and data not shown) and *CYS3* loci (data not shown). Then we examined DSB formation and processing in the *RAD50* context, under non-denaturing conditions, using a *PstI* digest and the *ARG4* EcoRV-BglII internal fragment as a probe. As previously reported for the *HIS4-LEU2* construct (Bishop et al. 1992; Shinohara et al. 1992; Schwacha and Kleckner 1997), we also observed that DSBs form at the *ARG4* (Figure 6A) and *CYS3* loci (data not shown) in the *rad51*, *rad55*, and *rad57* diploids and that for each mutant the DSB fragments are more heterogeneous in size than those in wild-type cells (*RFA1*). Using the same restriction digest, we were not able to detect DSB fragments at the *ARG4* locus in DNA prepared from *rad52* diploids. This is probably because the 5′ ends of the DSB fragments are extensively and rapidly degraded beyond the *PstI* site in the absence of the Rad52 protein, which renders the single-stranded DSB fragments resistant to cleavage by the restriction enzyme used to digest the genomic DNA, as described previously (White and Haber 1990).

To confirm this hypothesis, we used an *MluI-NruI* digestion instead of a *PstI* digest to obtain a parental genomic fragment of larger size covering the *ARG4* locus and used an internal fragment of the *YHR015* gene as a probe (see Figure 2, A and B). By using this restriction digest, we could consistently detect DSB signals as a faint smear that accumulates over the course of meiosis; the smallest fragments in the smear extend far below the uniconsented fragment observed in the *rad52 rad50S* diploid (Figure 6B). A comparison of the width of the DSB smear in the *rad51*, *rad55*, and *rfa1-t11* mutants was performed in the same experiment, with DNA from a *dmc1* mutant diploid included as a specific control for the extent of meiotic DSB degradation (Figure 6A). This comparison, which was performed four times, suggests that the range of fragment sizes is the most restricted in the *RFA1* and *rfa1-t48* diploids, intermediate in the *rfa1-t11*, *rad51*, *rad55*, *rad57*, and *dmc1* diploids, and the most extensive in the *rad52* diploid. To confirm this conclusion, we examined the fate of the 3′ single-stranded DNA end in the *rad51*, *rad52*, *rad55*, and *rad57* mutant strains on denaturing alkaline
Figure 6.—Detection of meiotic DSBs at the ARG4 locus in different mutant strains. (A) Samples of genomic DNA were extracted from RFA1 (ORD2130), rad51 (ORD3200), rad55 (ORD3204), dmc1 (ORD3233), rad57 (ORD3243), and rfa1-t11 (ORD3256) meiotic cells at the indicated times. DNA was digested with PstI and probed with the internal EcoRV-BglII fragment of ARG4. (B) Samples of genomic DNA were extracted from rad52 rad50S (ORD3284) and rad52 (ORD3285) strains, digested with MluI and NruI, and probed with a part of YHR015 as a probe. The positions of the parental and DSB fragments are indicated by horizontal arrows.

gels by using a single-stranded probe to detect ARG4 DSB fragments. In all cases, as for the RFA1 strain, we detected a meiosis-specific band of the expected size for the unresected 3' single-stranded fragment (Figure 5 and data not shown). Noticeably, at late time points (24 hr and more), a fragment of higher molecular weight than that of the parental fragment is apparent in the rad51, rad55, and rad52 diploids (Figure 5, open arrow). This band, never observed in RFA1 and rad50S diploids, likely reflects the fraction of single-stranded DSB molecules that contain an undigested MluI restriction site downstream of the DSB site (see Figure 5). This therefore confirms that DSBs in these mutants undergo hyperresection, as seen under non-denaturing conditions (Figure 6). Close examination of the hybridization signals of the expected size for this higher molecular weight intermediate in all of our experiments involving the other mutants (Figure 5 and data not shown) indicates that the rfa1-t11 strain sometimes exhibits a faint signal but that the rad57 and rfa1-t48 strains never do. These additional observations reinforce the conclusion that DSBs undergo extended resection in the rfa1-t11 mutant, to a greater extent than in the rfa1-t48 mutant (Figure 4), and that there is a subtle difference between the rad55 and rad57 mutants with respect to DSB processing. Altogether, we conclude that, as seen for RFA1 cells, all of the mutant diploids studied here form the unresected 3' single-stranded tails that are likely involved in the invasion of a homologous chromosome. However, all the mutants exhibit a more or less extensive resection of the 5' strand.

DISCUSSION

Over the past years, the importance of the RPA heterotrimer in numerous aspects of DNA metabolism has become very compelling. Indeed, in vivo and in vitro studies have demonstrated its involvement in replication
(for review, see Wold 1997), in repair (Coverley et al. 1991; Guzder et al. 1995), in mitotic recombination (Longhese et al. 1994; Firmenich et al. 1995; Smith and Rothstein 1995, 1999; Umezu et al. 1998), and in surveillance mechanisms that link defects in DNA metabolism with cell cycle progression (Brush et al. 1996; Cheng et al. 1996; Lee et al. 1998). The isolation of a number of mutants has been instrumental in the characterization of the multiple functions of the RPA complex (Longhese et al. 1994; Firmenich et al. 1995; Smith and Rothstein 1995, 1999; Umezu et al. 1998; Smith and Rothstein 1999). The present study of two of these rfa1 mutants adds insights into the role of RPA in meiotic recombination. We show here that homozygous rfa1-t11 and rfa1-t48 diploid strains have a low efficiency of sporulation and poor spore viability and that they are defective in the formation of recombinants. Physical analyses of early events in recombination indicate that both mutants form normal levels of meiosis-specific DSBs and produce 3′ single-stranded tails, indicating that the RPA complex present in these rfa1 mutants does not impair the formation of this key early recombination intermediate. However, DSBs undergo extensive resection in both rfa1 mutants. Similar phenotypes are observed for strains with mutations in the RAD52 pathway and in the double mutants (rad51 rfa1-t11, rad55 rfa1-t11, rad57 rfa1-t11, and rad52 rfa1-t11; data not shown). The similarity of the phenotypes conferred by the rfa1 mutations and the RAD52 pathway mutations, in particular an accumulation of hyperresected DSB fragments, the formation of a residual level of recombinant molecules but a stronger defect in the formation of mature recombinant cells (Bishop et al. 1992; Shinohara et al. 1992; Ogawa et al. 1993a), suggests that these rfa1 mutants are impaired in the formation of ssDNA nucleofilaments and/or in other post-DSB steps of homologous recombination.

Potential role of RPA in the formation of a proper ssDNA nucleofilament: The mechanistic steps by which ssDNA-binding proteins act in recombination were originally described through studies of the E. coli SSB protein (for reviews, see Meyer and Laine 1990; Kowalczykowski et al. 1994). More recent studies with eukaryotic proteins showed that the strand exchange reaction catalyzed by Rad51 proteins is rather inefficient in vitro but is enhanced when the cofactors RPA, Rad52, and the Rad55/Rad57 proteins are added in the proper order and stoichiometry (Sung 1994, 1997a, b; Benson et al. 1998; New et al. 1998; Shinohara and Ogawa 1998; for a review, see Sung et al. 2000). A possible explanation for the requirement of the eukaryotic Rad51 proteins for those cofactors may stem from observations that, in contrast to RecA, Rad51 proteins can bind both double-stranded DNA (dsDNA) and ssDNA (Shinohara et al. 1992; Benson et al. 1994) and that RPA competes with Rad51 protein for binding ssDNA. Any interpretation of the phenotype of rfa1 mutations should therefore consider the dual role of RPA in presynapsis and synopsis and should specifically take into account the role of the Rad52 and Rad55/Rad57 proteins as cofactors.

Two major biochemical functions of RPA could be differentially affected in the rfa1 mutants described here: the ssDNA-binding activity and/or interactions with other proteins. Considering the first possibility, we initially envisaged that RPA could interact in vivo with 3′ single-stranded DNA, and we therefore examined in great detail this key recombination intermediate in the rfa1 mutants. We found that both mutants form the resected intermediate with the same timing and strand polarity (3′ single-stranded tail) as does the RFA1 strain. This result suggests that RPA has no role in the formation or in the stabilization of the DSB single-stranded tail. In this case other DNA-binding proteins such as Rad52 or the Rad55/57 heterodimer might substitute for RPA in coating the single-stranded DSB tail. Although this extreme hypothesis cannot be formally excluded, the in vitro properties of RPA, the abundance of the RPA complex throughout meiotic prophase (Plug et al. 1997, 1998), and the induction of DSB-dependent recombination foci that contain RPA (Gasior et al. 1998, 2001) argue that RPA has a role in the early steps of recombination. A hypothesis that may explain why RPA complexes containing the rfa1-t48 mutation, localized within the conserved Rfa1-A ssDNA-binding domain (Philipova et al. 1996), retain ssDNA-binding activity is that RPA has four ssDNA-binding domains, an arrangement reminiscent of the tetrameric structure of SSB (Philipova et al. 1996). Another missense mutation (rfa1-D228Y) localized near the rfa1-t48 mutation (amino acids 228 and 221, respectively) has been previously described (Smith and Rothstein 1995). This mutation confers a slow growth phenotype, UV but not gamma irradiation sensitivity, and a slightly reduced efficiency of heteroallelic recombination. Biochemical analysis of the RPA complex present in rfa1-t48/Y strains indicates that the complex binds ssDNA but that its overall level is reduced twofold (Smith and Rothstein 1995). A tentative interpretation of the results obtained with the rfa1-t48/Y strain, similar to what was concluded for the rfa1-D228Y mutant, is that the Rfa1-t48 protein complex binds DNA but interacts abnormally with it, thereby promoting subsequent defects in DSB processing and repair.

Alternatively, the rfa1 mutants with alterations in the N-terminal part of the protein might be defective in interacting with other proteins. Two such rfa1 alleles with overlapping phenotypes, rfa1-t44 (G77D; Firmenich et al. 1995) and rfa1-t11 (K45E; Umezu et al. 1998), have been studied. Specifically, strains bearing these mutations are proficient for DNA replication, sensitive to UV and gamma irradiation, and strongly deficient in HO-induced recombination. The homozygous diploids have a reduced sporulation efficiency as well as severe
spore inviability (Firmenich et al. 1995; present results). A biochemical analysis of the purified RPA complex containing the Rfa1-t11 subunit indicates that it binds to ssDNA, but that Rad51 displaces the mutant RPA complex more slowly than the wild-type RPA complex and thereby impedes strand exchange (S. Kowalczykowski, personal communication). These mutations are localized in less conserved regions of the Rfa1 protein and are therefore more likely to be involved in species-specific protein-protein interactions. Evidence for this possibility is suggested by the observation that the mitotic phenotypes of rfa1-44 cells are suppressed in a dose-dependent manner by RAD52 (Firmenich et al. 1995). Studies of the interactions among the proteins of the RAD52 pathway (by two-hybrid assay and co-immunoprecipitation analyses) indicates that RPA interacts with Rad52, which in turn interacts with Rad51. This scenario raises the possibility that the primary defect of the rfa1-t11 mutant resides in the interaction of RPA with the Rad52 mediator protein (Hays et al. 1995, 1998; Park et al. 1996; Shinohara and Ogawa 1998). A defective interaction between Rfa1-t11 ssDNA and Rad51, which implicates the intermediation of Rad52, could result in a defect in the polymerization of Rad51 on ssDNA, thereby inhibiting the strand exchange reaction.

Potential role of RPA in post-ssDNA nucleofilament formation step(s): Once the ssDNA filament is properly formed, pairing between this presynaptic filament and a homologous dsDNA takes place and is followed by strand exchange, leading to the formation of a D-loop intermediate (for reviews, see Kowalczykowski et al. 1994; Sung et al. 2000). In vitro, the factors that function in the synaptic phase are the Rad51 ssDNA filament, the dsDNA, RPA, and the Rad54 and Rhh54/Tid1 proteins, which stimulate the formation of the D-loop by mediating an alteration of the duplex DNA conformation (Petukhova et al. 1998, 1999, 2000; Tan et al. 1999; Song and Sung 2000; VanKomen et al. 2000). Mutant analyses showed that the conversion of these intermediates to a double Holliday junction is dependent on the activity of the RAD51, RAD52, RAD55, RAD57, and DMC1 gene products (Schwacha and Kleckner 1997). Strains with mutations in all of these genes, as well as our rfa1 mutants, exhibit hyperresection of the 5' ends of the break fragments (Bishop et al. 1992; Shinohara et al. 1992, 1997; Dresser et al. 1997; Schwacha and Kleckner 1997; present study, Figures 4 and 6). The origin of this hyperresection is not known. It may reflect an impairment in the initial mechanism of resection, which itself could be coupled to DSB formation and the assembly of a proper ssDNA recombination intermediate for strand invasion. Alternatively, these events may be uncoupled and therefore hyperresection would be due only to an independent mechanism of degradation that is activated when DSB repair is defective. This observation favors the view that this hyperresection is the consequence of the defect in repair, which of course could be due to the uncoupling of degradation and strand exchange but could also result from the improper assembly of an active Rad51 nucleofilament.

In conclusion, our study of the rfa1-t11 and rfa1-t48 mutants has further revealed the involvement of the RPA complex in the process of meiotic recombination. A future interest is to define the biochemical properties of the corresponding RPA complexes with respect to the formation and the activity of the single-stranded DNA nucleofilament in strand invasion, a key step in the repair of DNA double-stranded breaks by homologous recombination.

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LITERATURE CITED


Brill, S. J., and B. Stillman, 1989 Yeast replication factor-A func-


Sung, P., 1997b Yeast Rad52 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes Dev. 11: 1111–1121.


