Genetic Analysis of Yeast RPA1 Reveals Its Multiple Functions in DNA Metabolism

Keiko Umezu,*,†,1 Neal Sugawara,‡ Clark Chen,*,†,2 James E. Haber‡ and Richard D. Kolodner*,†

**Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, 02115, and †Charles A. Dana Division of Human Cancer Genetics, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, and ‡Rosenstiel Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02254-9110*

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ABSTRACT

Replication protein A (RPA) is a single-stranded DNA-binding protein identified as an essential factor for SV40 DNA replication *in vitro*. To understand the *in vivo* functions of RPA, we mutagenized the *Saccharomyces cerevisiae RFA1* gene and identified 19 ultraviolet light (UV) irradiation- and methyl methane sulfonate (MMS)-sensitive mutants and 5 temperature-sensitive mutants. The UV- and MMS-sensitive mutants showed up to 10^4 to 10^5 times increased sensitivity to these agents. Some of the UV- and MMS-sensitive mutants were killed by an HO-induced double-strand break at *MAT*. Physical analysis of recombination in one UV- and MMS-sensitive *rfa1* mutant demonstrated that it was defective for mating type switching and single-strand annealing recombination. Two temperature-sensitive mutants were characterized in detail, and at the restrictive temperature were found to have an arrest phenotype and DNA content indicative of incomplete DNA replication. DNA sequence analysis indicated that most of the mutations altered amino acids that were conserved between yeast, human, and Xenopus RPA1. Taken together, we conclude that RPA1 has multiple roles *in vivo* and functions in DNA replication, repair, and recombination, like the single-stranded DNA-binding proteins of bacteria and phages.

REPLICATION Protein A [RPA, also known as Replication Factor A (RFA) or human SSB],[3] is a single-stranded DNA- (ssDNA-) binding protein that was initially identified as an essential factor for replication of SV40-ori containing DNA *in vitro* and is likely to be the eukaryotic equivalent of the *Escherichia coli* SSB protein required for most aspects of bacterial DNA metabolism (Stillman 1989; Tsurimoto *et al.* 1989; Kenny *et al.* 1990; Collins and Kelly 1991; Lee *et al.* 1991; Dornreiter *et al.* 1992; Bochkarev *et al.* 1997). Numerous biochemical studies have supported this point of view. Analysis of RPA in the SV40 replication system revealed that it is involved in both the initiation and elongation phases of DNA replication (Stillman 1989; Tsurimoto and Stillman 1989; Kenny *et al.* 1990; Collins and Kelly 1991; Lee *et al.* 1991; Dornreiter *et al.* 1992). RPA is also involved in other aspects of DNA metabolism. RPA is required for *in vitro* reconstitution of nucleotide excision repair (Coverley *et al.* 1991; Aboussekhra *et al.* 1995; Kazantsev *et al.* 1996) and its physical association with and functional stimulation of XPA, XPG, and XPF, along with its ability to bind to UV-damaged DNA, further confirm the importance of RPA in nucleotide excision repair (He *et al.* 1995; Lee *et al.* 1995; Li *et al.* 1995; Matsuda *et al.* 1995; Burns *et al.* 1996; Matsunaga *et al.* 1996). Antibody depletion and *in vitro* reconstitution studies have demonstrated that RPA is required for mismatch repair (Lin *et al.* 1997). Finally, RPA stimulates the *in vitro* strand exchange activities of *Saccharomyces cerevisiae* SEP1 (Strand Exchange Protein), human HPP1 (Homologous Pairing Protein), and RAD51 (a eukaryotic homologue of the *E. coli* RecA) (Heyer *et al.* 1990; Moore *et al.* 1991; Alani *et al.* 1992; Shinohara *et al.* 1992; Ogawa *et al.* 1993; Sung 1994, 1997; Sugiyama *et al.* 1997), and human RPA physically interacts with the human *RAD52* gene product via RPA2 (Park *et al.* 1996) consistent with a role in genetic recombination. In sum, good biochemical evidence exists that RPA plays a crucial role in DNA replication, repair, and recombination.

RPA is an evolutionarily conserved protein consisting of three subunits: RPA1 (*Mr* 70,000), RPA2 (*Mr* 36,000), and RPA3 (*Mr* 14,000). Of these three subunits, RPA1 is the best characterized biochemically. While most biochemical characterization of RPA1 has been done using human RPA1, extrapolation of these results to other eukaryotic RPAs seems justified given the high degree

Corresponding author: Richard Kolodner, Ludwig Institute for Cancer Research, UC San Diego School of Medicine, CMME-3080, 9500 Gilman Drive, La Jolla, CA 92093-0660.
E-mail: rkolodner@ucsd.edu

[1] *Present address:* Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Nara 630-01, JAPAN.

[2] *Present address:* Ludwig Institute for Cancer Research, UC San Diego School of Medicine, La Jolla, CA 92093-0660.

[3] Replication Protein A has been given several different names. In this article, the protein and protein subunits are called RPA as this is the first name we associate with the protein. Genes and mutant alleles are called *RFA* and *rfa*, respectively, as this is the official designation in *Saccharomyces cerevisiae*.

of conservation among the various RPA homologues (Heyer *et al.* 1990; Brill and Stillman 1991; Erdile *et al.* 1991; Adachi and Laemmli 1992; Brown *et al.* 1994; Ishiai *et al.* 1996). Functional dissection of human RPA1 has identified three distinct domains. The N-terminal 100 amino acids of human RPA1 are required for stimulation of DNA polymerase alpha (Kim *et al.* 1996). The ssDNA-binding activity of human RPA1 resides between amino acid residues 175–420 (Gomes and Wold 1995; Kim *et al.* 1996; Lin *et al.* 1996). X-ray crystallographic analysis of this region suggested that it is comprised of two structurally homologous subdomains oriented in tandem (Bochkarev *et al.* 1997). The overall structure of the RPA1 subdomains appears similar to those of previously solved ssDNA-binding proteins, T4 gp32 and f1 gene V protein (Bochkarev *et al.* 1997). The C-terminal 100 amino acids of human RPA1 are required for binding RPA2 and RPA3 to form the RPA heterotrimer (Gomes and Wold 1996; Lin *et al.* 1996). Located within the C-terminal third of RPA1 is a putative C4 zinc finger motif conserved among all eukaryotic homologues (Erdile *et al.* 1991; Adachi and Laemmli 1992; Brown *et al.* 1994; Ishiai *et al.* 1996), and this region of RPA1 appears to be required for DNA replication and mismatch repair but not nucleotide excision repair (Lin *et al.* 1996; Lin *et al.* 1997).

While RPA1 alone binds to ssDNA with high affinity (Brill and Stillman 1989; Wold *et al.* 1989; Kenny *et al.* 1990), the complete holocomplex is required for DNA replication *in vitro* (Erdile *et al.* 1991). Consistent with this observation, all three subunits of RPA are required for viability in *S. cerevisiae* (Heyer *et al.* 1990; Brill and Stillman 1991). Although the functions of RPA2 and RPA3 remain to be elucidated, recently it was shown that the minimal regions of RPA2 and RPA3 necessary to maintain viability in *S. cerevisiae* bear weak sequence similarity to the *E. coli* SSB and to the two ssDNA-binding subdomains of RPA1. Based on this observation, it was proposed that the four ssDNA-binding subdomains of RPA function like the homotetrameric *E. coli* SSB (Maniar *et al.* 1997). Consistent with this hypothesis is the observation that *S. cerevisiae* RPA2 has contacts with ssDNA (Philipova *et al.* 1996). Also consistent is the isolation of *S. cerevisiae RFA2* and *RFA3* alleles that are defective in DNA replication (Santocanale *et al.* 1995; Maniar *et al.* 1997). In addition to a role in ssDNA binding, RPA2 has been implicated as the regulatory subunit of the RPA heterotrimer (Din *et al.* 1990; Dutta and Stillman 1992; Fotedar and Roberts 1992; Cardoso *et al.* 1993; Liu and Weaver 1993; Carty *et al.* 1994).

While there is considerable biochemical data suggesting that RPA functions in a variety of DNA metabolic processes, only limited genetic analysis has been performed to elucidate its roles *in vivo*. Initial studies using *rfa1* null alleles reported that *RFA1* is an essential gene and demonstrated that *rfa1* mutations caused a terminal arrest phenotype consistent with a defect in DNA replication (Heyer *et al.* 1990; Brill and Stillman 1991). One subsequent study reported the construction of two-amino-acid insertion mutations in *RFA1*, some of which caused weak temperature sensitivity, weak UV-sensitivity, and a modest defect in mitotic intrachromosomal recombination (Longhese *et al.* 1994). Another study reported an *rfa1* mutation (*rfa1-44*) that caused a defect in HO-endonuclease-induced plasmid-to-chromosome gene conversion and sensitivity to UV and X rays (Firmenich *et al.* 1995). Overexpression of RAD52 suppressed the *rfa1-44* mutation, providing evidence that RPA and RAD52 interact (Firmenich *et al.* 1995). Subsequent studies of mating type switching using physical assays of recombination detected only an apparant 50% failure in recombination caused by the *rfa1-44* allele (N. Sugawara and J. E. Haber, unpublished data). A third *rfa1* mutation was isolated in a screen for suppressors of the defect in direct repeat recombination in *rad1 rad52* double mutants, and this *rfa1* allele by itself was found to cause some defects in genetic recombination (Smith and Rothstein 1995). Other studies of *RFA1* have suggested a role for RPA in checkpoint control (Brush *et al.* 1996; Longhese *et al.* 1996; Parker *et al.* 1997) and have suggested that RPA could serve as a DNA damage sensor (Brush *et al.* 1996).

While previous studies described the first *rfa1* mutants, they either screened for a single phenotype and identified a single mutant (Firmenich *et al.* 1995; Smith and Rothstein 1995), or they isolated a small number of mutants and then tested them for significant phenotypes (Heyer *et al.* 1990; Brill and Stillman 1991; Longhese *et al.* 1994). Consequently, they may not have been capable of revealing the wide variety of metabolic defects that could be caused by mutations in the *RFA1* gene. In the present study, we focused on randomly mutagenizing the *RFA1* gene and systematically screened for mutations that confer a temperature-sensitive (ts) and UV- or MMS-sensitive phenotype in order to isolate a variety of alleles. We isolated 24 *rfa1* alleles and characterized 19 DNA-damage-sensitive mutants and two ts mutants in greater detail.

MATERIALS AND METHODS

Media: *E. coli* strains were grown in luria bertani (LB) medium (Miller 1972), which was supplemented with 100 μg/ml ampicillin when required. SOC medium (Sambrook *et al.* 1989) was used for incubation of the cells after electro/transformation. Complex glucose [yeast extract reptone adenine dextrose (YPD)], sporulation, synthetic complete (SC), and various drop-out media for growth of yeast strains were made as previously described (Sherman *et al.* 1983). Adenine sulfate was added to YPD at 0.004% (YPAD) when indicated. 5-Fluoro-orotic acid (5-FOA) was used as described (Rose *et al.* 1990). Plates containing Methyl Methane Sulfonate (MMS, Kodak, Rochester, NY) were used within two days after preparing them. For HO-induction experiments designed to test for sensitivity to HO expression, cells were grown in SC medium

lacking glucose, uracil, and leucine and containing 3% glycerol and 2% sodium lactate (pH 5.5). To induce the GAL-HO gene, galactose (2%) was added to liquid cell cultures grown in this medium, or the culture was spotted onto solid medium lacking glucose, uracil, and leucine and containing 2% galactose. For analysis of HO-induced mating type switching, cells were propagated in yeast extract peptone-lactate medium and induced with galactose as previously described (Connolly *et al.* 1988).

S. cerevisiae* and *E. coli* strains:** Strain YSB89 (*MATa**/*MAT*α *ade2-1*/*ade2-1 ura3-1*/*ura3-1 his3-11,15*/*his3-11,15 trp1-1*/*trp1-1 leu2-3,112*/*leu2-3,112 can1-100*/*can1-100 rfa1::TRP1*/*RFA1*) derived from the parental strain W303 was kindly provided by Dr. B. Stillman (Cold Spring Harbor Laboratory) (Brill and Stillman 1991). Strains RKY2102 [*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rfa1::TRP1* (pRPA1 *URA3 RFA1*)] and RKY1900 [*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rfa1::TRP1* (pKU1 *LEU2 RFA1*)] were constructed by transforming YSB89 with plasmids pRPA1 and pKU1 (see below), respectively, sporulating the strain, and then identifying an appropriate spore clone after tetrad dissection. The isogenic wild-type strain W303-1A was obtained as a spore clone from YSB89. To obtain the strain used for analysis of single-strand annealing, the W303 strain containing the *rfa1-t11* allele was crossed to tNS1373M [*ho HML*α *leu2 MAT*α*-inc hmr-3*Δ *mal2 trp1 thr4 GAL+ ura3-Nco-pUC-HOcs-*λ*H3(2.3 kb)-URA3 GAL::HO* at *THR4*] (Sugawara and Haber 1992) to obtain *rfa1-t11* segregants that were *ura3 leu2 mat*α*-inc* and possessed *GAL-HO* integrated at the *THR4* locus. The resulting strain was then transformed with pNSU208 (see below). The *E. coli* strains DH5 and DH5α were used to amplify and manipulate all plasmids described in this study.

Plasmids: The plasmid pRPA1 (Heyer *et al.* 1990) contains a 3.4-kb *Sau*3A insert carrying the *RFA1* gene in the *Bam*HI site of the *ARS1 CEN4 URA3* vector YCp50. Plasmid pKU1 was constructed by subcloning the 3.1-kb *Sal*I-*Hin*dIII fragment containing the *RFA1* gene from the plasmid pRPA1 into the corresponding sites of the *ARSH4 CEN6 LEU2* vector pRS415. A series of plasmids for *rfa1* allele replacement pKU2 was constructed by inserting the 3.1-kb *Sal*I-*Bam*HI fragment of pKU1 derivatives carrying the *rfa1* mutant alleles into the corresponding sites of the *URA3* integration plasmid pRS406. Plasmids pKU1 and pKU2 carrying the *rfa1* mutants are indicated by hyphenating allele names. The *GAL-HO* plasmids pJH132 (*GAL-HO URA3 ARS1 CEN4*) and pJH727 (*GAL1-HO LEU2 ARS1 CEN4*) have been described (Jensen and Herskowitz 1984; White and Haber 1990). The plasmid pNSU208 was constructed by inserting the *Eco*RV-*Bss*HII fragment from the *lacZ* sequence of pJF3 into the *Hin*dIII site of pNR16 (Fishman-Lobell *et al.* 1992).

Genetic techniques: *E. coli* strains were transformed with DNA by electroporation using Gene Pulser (Bio-Rad, Richmond, CA). Standard methods for yeast genetics were essentially according to Rose *et al.* (1990). Transformation of yeast cells was performed using the lithium acetate procedure as described (Gietz *et al.* 1992). Plasmid DNA was isolated as described (Rose *et al.* 1990). Allele replacement was performed by standard methods involving transformation with pKU2 series plasmids that had been linearized at a site in the *RFA1* gene by digestion with *Nhe*I.

***RFA1* mutagenesis:** Mutagenesis of the *RFA1* gene was performed by PCR based on the technique of Muhlrad *et al.* (1992), except that standard PCR conditions were used. The error rate of *Taq* polymerase is in the range of 0.1×10^{-4} to 2×10^{-4} per nucleotide under standard PCR conditions (Cadwell and Joyce 1994), and at this error rate we estimated that 12 cycles of standard PCR over a region of about 2 kb of the *RFA1* open reading frame (ORF) was sufficient to generate a diverse mutation library. PCR primers were designed to amplify the 2.8-kb region of pKU1 containing the *RFA1* open reading frame and the flanking sequences on both sides of the open reading frame. The primers 5′-ATGGTGCATGCA AGGAGATGGC and 5′-CACTTTATGCTTCCGGCTCCTA, which were synthesized by Molecular Biology Core Facility, Dana-Farber Cancer Institute, Boston, MA, were complementary to vector sequences located 180 nucleotides upstream and downstream from the unique *Mlu*I and *Hin*dIII sites in the vector, respectively. The *Mlu*I and *Hin*dIII sites are located 165 bp upstream from the initiation codon and 252 bp downstream from the termination codon of the *RFA1* open reading frame, respectively. PCR mixtures (25 μl) contained 1.25 units of *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT), 10 ng of template DNA (pKU1 linearized at the unique *Cla*I site in the *LEU2* gene), 1 μM of each primer, 200 μM each dNTP, 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm $MgCl_2$, and 0.001% (w/v) gelatin. PCR was performed in a Perkin Elmer 9600 utilizing an initial incubation at 95° for 5 min, followed by 12 cycles of 30 sec at 95°, 30 sec at 45°, and 90 sec at 72°. Twenty independent reaction mixtures were combined (total 500 μl) and purified using a QIAquick Spin Column (QIAGEN, Santa Clarita, CA). The PCR product and the 6.8-kb *Mlu*I-*Hin*dIII fragment of pKU1 (the vector fragment of pKU1 obtained by digestion with *Mlu*I-*Hin*dIII, followed by purification by agarose gel electrophoresis to remove the 2.4-kb *RFA1*-containing fragment) were mixed in a 1:1 molar ratio and cotransformed into RKY2102. Transformants were selected on SC plates lacking Leu and containing Ura and then replica-plated onto 5-FOA plates to eliminate pRPA1 by plasmid shuffling (Boeke *et al.* 1987). These 5-FOA plates were used as master plates for screening of *rfa1* mutants. Selection and growth of transformants during this mutagenesis procedure were at 26°.

Screening of *rfa1* mutants: To screen candidate *rfa1* mutants for those having temperature-sensitive (ts) growth, UV-sensitive (UV^S), and MMS-sensitive (MMS^S) phenotypes, each 5-FOA master plate was replica-plated onto a set of four YPD plates; 7.5 μM of erythrosine B (Sigma, St. Louis, MO) was included in the plates to stain nonviable cells (Bonneu *et al.* 1991). In addition, MMS (0.01%) was included in one of these four plates. In some cases, adenine sulfate (YPAD) was added to suppress coloring due to the *ade2* mutation and to make the red staining by erythrosine B more visible (Bonneu *et al.* 1991). One of the YPD (or YPAD) plates was irradiated with 50 J/m^2 of UV using a Stratalinker 1800 (Stratagene, La Jolla, CA) and incubated at 26° along with the YPD-MMS plate and an untreated YPD plate that served as a control. In addition, one YPD plate was incubated at 37°. After incubation for 3 days, the growth and color of the colonies on the 37°, UV, and MMS plates were compared to the control plates, and the colonies showing a growth defect or reddish color were recovered as candidate mutants and streaked for single colonies on YPD or YPAD plates containing erythrosine B. A colony from each candidate mutant was retested using the semiquantitative assays described below.

Semiquantitative assays: Cells from a single colony were streaked on three YPD plates. One plate was incubated at 26° and served as a control. One plate was irradiated with 50 J/m^2 of UV and incubated at 26°. And the third plate was incubated at 37°. Cells from the same colony were also picked into sterile water and fourfold serial dilutions were prepared. Five-microliter aliquots of each dilution were spotted onto three YPD plates, one of which contained 0.01% MMS and another of which was irradiated with 50 J/m^2 of UV. These three plates were incubated at 26°. After incubation for 2 days, the plating efficiency and extent of the growth on each plate compared to the control plates were classified as follows: for UV and

MMS sensitivity: s, 10- to 10^2-fold decrease in plating efficiency; ±, decreased <10-fold. For temperature-sensitive growth: ss, no or little growth; s, very small colonies (<10% the size of the colonies on the control plates); ±, smaller colonies (10 to 50% the size of the colonies on the control plates).

Quantitative UV survival tests: Overnight cultures were inoculated into liquid YPAD and grown to early logarithmic phase (2–5 × 10^6 cells/ml). The cells were harvested by centrifugation, suspended in sterile water, recentrifuged, and resuspended in sterile water at 1 × 10^6 cells/ml, followed by brief sonication to disrupt aggregates. Serial dilutions were prepared in sterile water, aliquots were spread on YPD plates, and the plates were UV-irradiated as indicated. The plates were then incubated at 26° in the dark for 4 days before counting colonies.

Quantitative MMS survival tests: Cells were prepared as described under quantitative UV survival tests to obtain a cell suspension containing 1 × 10^6 cells/ml. MMS was added to 0.5 ml of cell suspension at the indicated concentrations followed by incubation for 40 min at room temperature. An equal volume of freshly prepared 10% (wt/vol) NaS_2O_3 was added, and serial dilutions of the cells were prepared in sterile water and spread on YPD plates. Colonies were counted after incubation at 26° for 4 days.

Flow cytometry: Cells were grown in liquid YPAD to early logarithmic phase (2–5 × 10^6 cells/ml) at 26°, and then the culture was divided into two portions: one portion was incubated at 26° and the other portion was incubated at 37°. Cells were withdrawn at each indicated time point and prepared for fluorescence-activated cell sorting (FACS) analysis as previously described (Johnson and Kolodner 1995). The resulting samples were analyzed using a FACScan flow cytometer with the CellFIT cell cycle analysis program (version 2.01.2, Becton Dickinson, Franklin Lakes, NJ) by the Core Flow Cytometry Facility, Dana-Farber Cancer Institute. A portion of each sample also was examined by light microscopy.

DNA sequencing: The entire region corresponding to the *RFA1* open reading frame in mutant pKU1 plasmids was sequenced with *Taq* DNA polymerase and dye terminators using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) by Molecular Biology Core Facility, Dana-Farber Cancer Institute. Six sequencing primers were used and were synthesized by the Molecular Biology Core Facility: 5′-GGCGAAAC CAGCAAGAAGAC, 5′-CTCAGAGCATCCAAATGAAACC, 5′-GAAGCCAAAGTATACTATGTATC, 5′-TAAAGGTGTTCGT GTGACGGA, and 5′-ATTTTGCATATCCTGCCTGTTC were complementary to the transcribed strand of *RFA1* at nucleotides −39 to −20, 372 to −393, 751 to −773, 1131 to −1151 and 1701 to −1722, respectively, and 5′-CTCATATGTTACA TAGATTAAATAG was complementary to the nontranscribed strand of *RFA1* at nucleotides 1886 to 1911 (numbering is from the first base of the initiation codon, which is 1) (Heyer *et al.* 1990).

Physical analysis of mating type switching and single-strand annealing: Strains W303-1A and an isogenic derivative containing the UV^S, MMS^S *rfa1-t11* allele were transformed with pJH727 containing *GAL1::HO CEN4 LEU2*. Galactose inductions were carried out as described previously (Connolly *et al.* 1988). Briefly, the strains were grown to 10^7 cells/ml in yeast extract peptone-lactate medium. At time zero and subsequent time points, DNA was extracted from aliquots using a glass bead protocol (Connolly *et al.* 1988). The strains were induced by the addition of galactose (2%, w/v) for 30 min, followed by the addition of glucose (2% w/v) to down-regulate the expression of *GAL::HO*. At 0 hr and 5 hr aliquots of cells were diluted in sterile H_2O and plated on YPD. Colonies were later scored for their phenotypes. DNA samples were digested with *Sty*I, electrophoresed through agarose gels, and analyzed by Southern blotting using a *MAT*-distal probe (pJH364) (White and Haber 1990). A Molecular Dynamics (Sunnyvale, CA) PhosphorImager was utilized to measure band intensities. Single-strand annealing was assayed using the *rfa1-t11* derivative of tNS1373M essentially as described above for the physical analysis of mating type switching except for the following procedural modifications. Glucose was not added to down-regulate the expression of *GAL::HO*. The DNA was digested with *Eco*RI, blotted, and probed with the *Bsu*36-*Sac*I fragment from *lacZ* to detect the plasmid products of HO cleavage and single-strand annealing. Colonies were grown on YPD and replica-plated onto media lacking leucine to assay for plasmid loss after induction of HO endonuclease.

RESULTS

Isolation of *rfa1* mutants: We have attempted to systematically analyze the *in vivo* functions of RPA in DNA metabolism by screening for *rfa1* mutants that confer ts, UV^S, or MMS^S phenotypes. Ts mutants were selected to identify mutations causing defects in DNA replication. UV^S mutants were selected to identify mutations causing defects in nucleotide excision repair and/or recombinational repair. And MMS^S mutants were selected to identify mutations that cause defects in recombination as well as possibly defects in other types of DNA repair (Esposito and Wagstaff 1981; Petes *et al.* 1991). The mutants isolated in these screens have subsequently been tested for mutator phenotypes, and the results of these studies will be described elsewhere.

The yeast strain RKY2102 was cotransformed with *RFA1* fragments that had been amplified by PCR under mutagenic conditions and pRS415 derivative vector fragments, resulting in reconstruction of an *RPA1* plasmid by *in vivo* homologous recombination. We then screened 15,000 Leu$^+$ 5-FOA-resistant transformants for ts, UV^S, and MMS^S phenotypes, first using erythrosine B staining as a growth defect indicator (the first screening) and second using semiquantitative measurements of the expected phenotypes. Fifty-four candidate mutants were obtained for further study, some of which had more than one phenotype. No mutants were obtained in control experiments in which *RFA1* was not amplified by PCR prior to the transformation step.

The resulting candidate mutant plasmids were retested by isolating plasmid DNA from each mutant and transforming it back into RKY2102 followed by plasmid shuffling. Nineteen out of 22 of the UV^S and/or MMS^S mutant plasmids conferred a similar degree of sensitivity to that of the original mutants from which the plasmid had been isolated. The sensitivity of all 19 mutants was suppressed in the presence of pRPA1 carrying the wild-type *RFA1* gene, indicating that the mutants were all recessive. After plasmid rescue, only 5 out of 10 ts mutant plasmids tested conferred a ts phenotype. All 5 of these ts mutants were recessive to wild type. In total, 24 mutants were obtained and 21 were studied further; specific examples of the analysis of these mutants are presented below, and a summary of the properties of

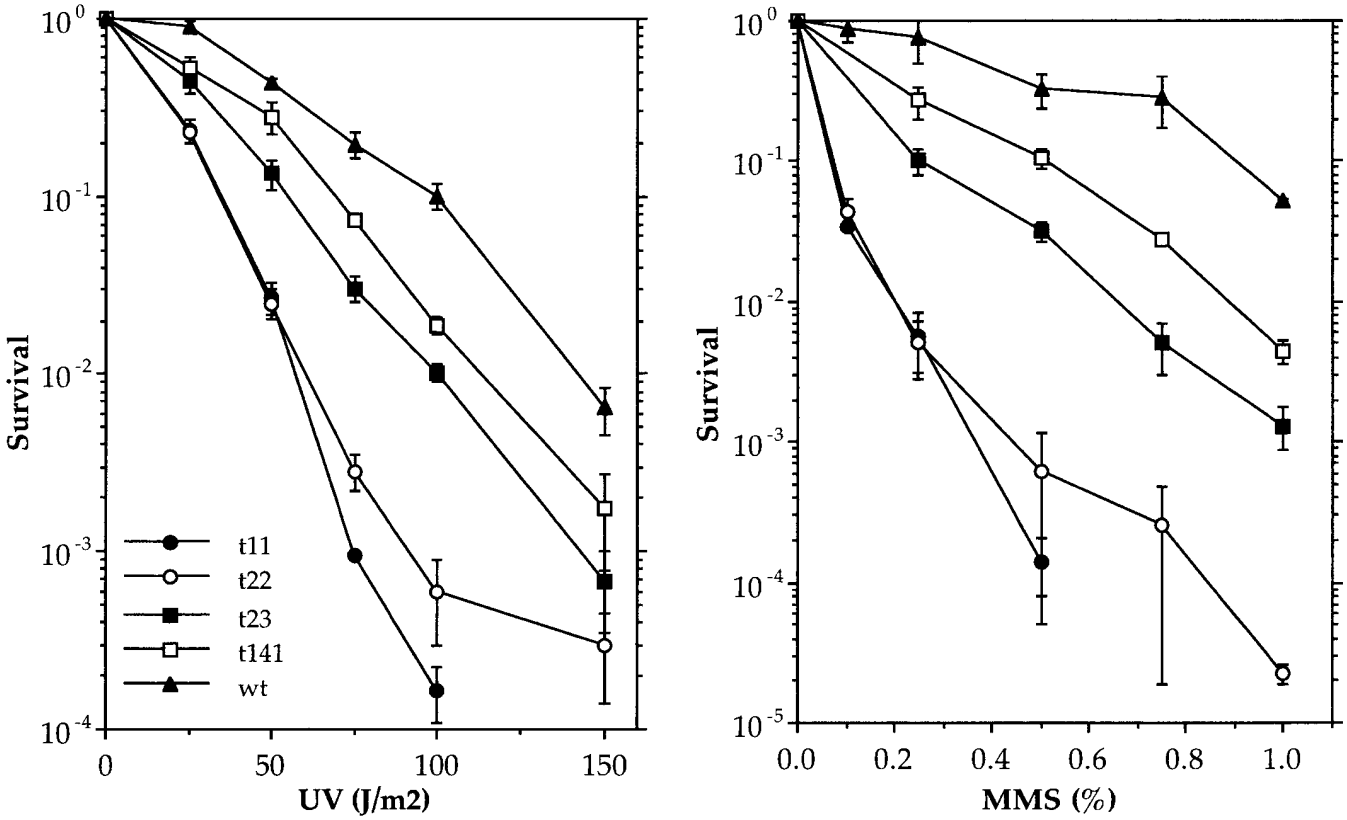


Figure 1.—Survival of representative mutants treated with either UV-irradiation or MMS. Quantitative UV and MMS survival tests were performed as described in materials and methods. The number shown next to each symbol indicates the mutant allele tested. The wild-type *RFA1* control strain, RKY1900, carries the *RFA1* gene on pKU1 and has the same *rfa1* deletion mutation on the chromosome as the *rfa1* mutant strains tested; all of the *rfa1* alleles examined were present on a plasmid in this experiment.

all of the mutants, including quantitative data, are presented in Figure 8 at the end of this article.

UV^S and MMS^S mutants: Nineteen mutants showing increased sensitivity to UV-irradiation and/or MMS were identified and analyzed in greater detail using quantitative tests. Figure 1 shows the survival curves obtained after treatment of representative mutants with a range of doses of either UV-irradiation or MMS, and the survival of each of these mutants after treatment with a fixed dose of either UV-irradiation or MMS is presented in Figure 8. All experiments were performed using *rfa1*Δ mutant strains (RKY2102 derivatives) in which the mutant *rfa1* allele of interest was present in the strain on an *ARS CEN* plasmid. In addition, control experiments were performed using RKY2102 and RKY1900 derivatives carrying the wild-type *RFA1* gene on an *ARS CEN* plasmid. Selected mutations were also transferred to the chromosomal *RFA1* locus and similarly analyzed to ensure that the plasmid-born alleles and chromosomal alleles behaved similarly.

All of the mutants obtained were sensitive to both UV-irradiation and MMS, even though the initial qualitative tests suggested the existence of mutants that were sensitive to killing by only one of the two agents. The mutants showed a wide range of sensitivity to both UV-irradiation and MMS at the doses tested; survival of mutants relative to the wild-type strain ranged from 10^{-3} to 0.5 for UV-irradiation and from 10^{-4} to 0.7 for MMS (Figure 8). There was a direct correlation between the degree of sensitivity to UV-irradiation and to MMS for each mutant. An interesting feature of the data is that the weakly and moderately UV^S alleles showed similar sensitivity to MMS, whereas the strongly UV^S alleles showed an even greater sensitivity to MMS; this is more apparent when the data presented in Figure 8 are reranked in order of increasing sensitivity.

The experiments discussed above (Figures 1 and 8) were performed with strains carrying the *rfa1* allele on an *ARS CEN* vector. Although we obtained the same results when either YPAD or Leu drop-out media that select for the marker on the plasmid (*LEU2*) was used, loss of the plasmid could conceivably have affected the results. To eliminate this possibility, the *rfa1-t11* allele was integrated at the chromosome *RFA1* locus, and the UV-sensitivity and MMS-sensitivity of the resulting strain were determined. Survival of this strain to killing by UV-irradiation and MMS was 3.85×10^{-4} and 1.02×10^{-4}, respectively, compared to the wild-type parental strain, which was similar to that obtained with *rfa1-t11* allele on an *ARS CEN* plasmid at the same doses of UV-irradia-

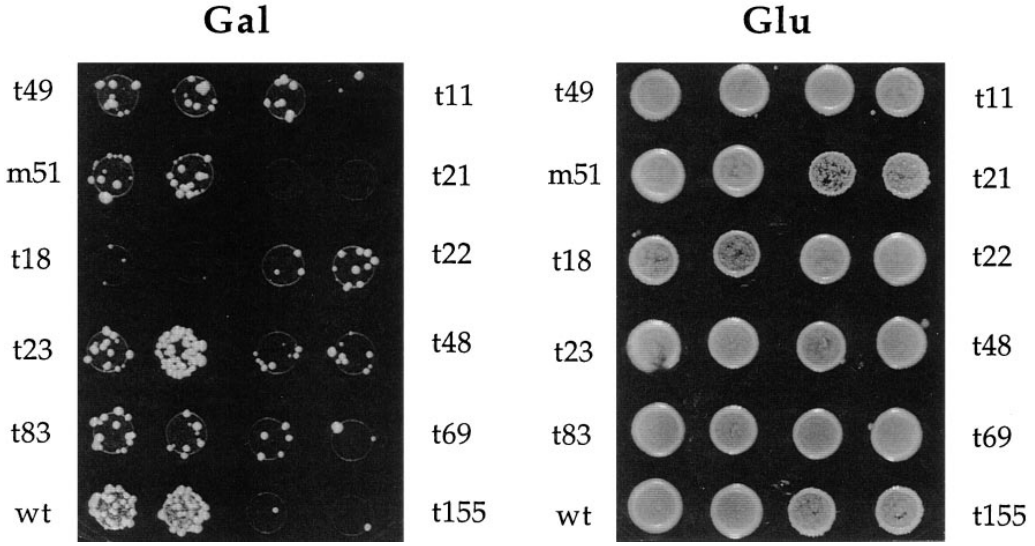


Figure 2.—Sensitivity of UVS and MMSS mutants to HO endonuclease. The indicated mutant strains transformed with the GAL-HO plasmid pJH132 were grown in SC medium lacking glucose, uracil, and leucine and containing 3% glycerol and 2% sodium lactate (pH 5.5) to early logarithmic phase. Approximately 7×10^4 cells from each culture were spotted in duplicate onto an SC plate lacking glucose, uracil, and leucine and containing 2% galactose to induce the GAL-HO gene (left panel) or were spotted onto an SC plate lacking uracil and leucine as control (right panel). The plates were then incubated at 26° for 4 days. As in Figure 1, RKY1900 was used as a wild-type control strain.

tion and MMS (Figure 8). Similar results were obtained with the *rfa1-t49* allele as well as three additional alleles that will be described elsewhere.

Some of the mutant strains, especially the strongly UV^S and MMS^S mutants, had an increased doubling time compared to the wild-type strains tested (Figure 8). This could be due to failure to repair endogenous DNA damage in these mutants resulting in triggering of a DNA damage checkpoint. Alternately, these mutant strains might also have defects in DNA replication that cause reduced growth rates. To examine these possibilities, we analyzed early logarithmic phase cells of strains carrying either the *rfa1-t11* chromosomal allele or the *rfa1-t11* plasmid allele using both FACS and light microscopy. Compared to the wild-type control strain (RKY2102), both mutant strains showed no significant increase in the proportion of cells present in a specific phase of the cell cycle such as S phase or G2 phase (data not shown). This suggests that the *rfa1-t11* allele does not cause a defect in DNA replication or trigger a DNA damage checkpoint.

Sensitivity of UVS and MMSS mutants to HO endonuclease: We anticipated that mutants that were sensitive to killing by both UV-irradiation and MMS might also have defects in genetic recombination because both UV-irradiation and MMS are known to produce damage in DNA that can be repaired by genetic recombination (Petes *et al.* 1991). To examine whether the UV^S and MMS^S mutants had such defects, we induced double-strand breaks at *MAT* by galactose induction of HO-endonuclease and determined whether the mutants were killed by this treatment; the mutants showing very weak UV^S and MMS^S phenotypes were not tested. Two different types of tests were performed: one measured the plating efficiency of the mutants on plates containing galactose and the other measured the effect of transient induction of HO on survival. These tests allowed the identification of three classes of mutants.

The transformation efficiency of mutants carrying the *rfa1-t21*, *rfa1-t155*, and *rfa1-t18* alleles with pJH132 (p*GAL-HO*) was much lower than with the other strains examined when the transformants were selected on plates containing glucose. The pJH132-transformed cells had growth defects even on glucose plates without galactose and were unable to survive on galactose plates (Figure 2). This suggests that these mutants were extremely sensitive to double-strand breaks and that either the extremely low level of HO expression in the presence of glucose is sufficient to kill some of the cells or that HO expression is increased in these *rfa1* mutants.

Strains carrying the *rfa1-t11*, *rfa1-t22*, *rfa1-t48*, and *rfa1-t69* alleles and the p*GAL-HO* plasmid showed clear differences in growth in the presence and the absence of galactose (Figure 2). These strains grew somewhat more slowly than a wild-type strain on glucose plates, and the survival of these mutant strains on galactose plates was much lower than the wild-type control strain. The strain carrying the *rfa1-t11* allele was examined in greater detail by exposing the strain to galactose for different periods of time and then plating the cells onto glucose plates to measure survival. Ninety percent of the cells carrying the *rfa1-t11* allele were killed after 4 hr of exposure to galactose compared to the wild-type control strain, confirming the extreme sensitivity of this

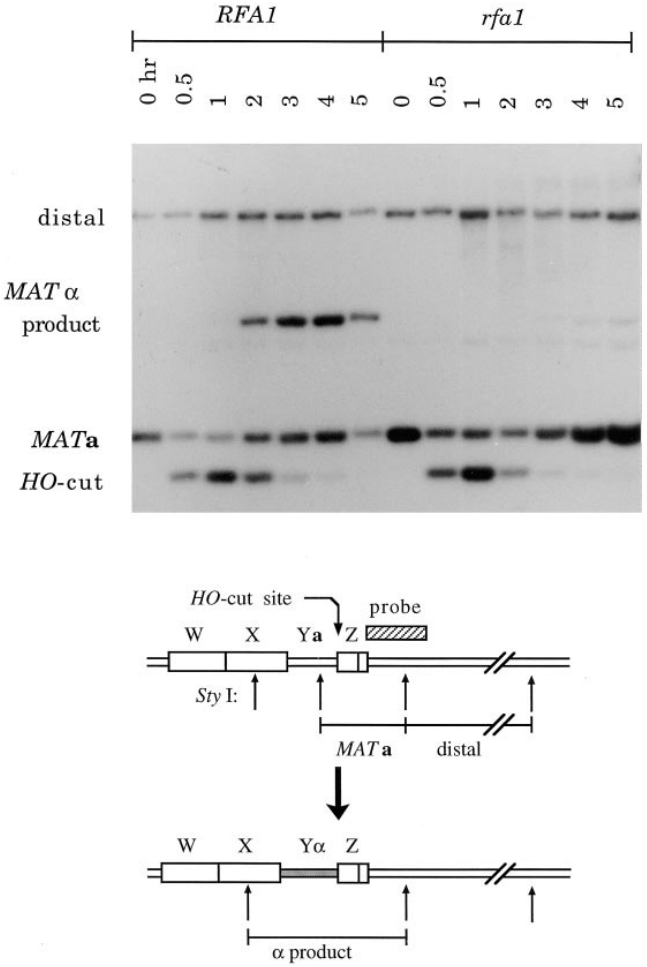


Figure 3.—*RFA1* is required for mating type switching after induction of HO endonuclease. An *rfa1-t11* mutant containing the *rfa1* allele at the chromosomal locus and a wild-type strain were induced to switch mating type by expression of a *GAL::HO* fusion. DNA was extracted at the time points shown, digested with *Sty*I, electrophoresed under neutral conditions, blotted and hybridized with a *MAT*-specific probe (hatched box). At 0 hr the probe hybridizes to the *MAT***a** fragment (0.93 kb) and the MAT_{distal} fragment (4.3 kb). After *GAL::HO* expression a smaller *HO*-cleaved fragment (0.72 kb) appears, followed at later time points by the appearance of a *MAT*a product band (1.88 kb). This band is larger because it lacks a *Sty*I site in the Ya sequence that is present in Y**a.** In the *rfa1* mutant the product band is faint but detectable.

mutant to even transient induction of double-strand DNA breaks at *MAT* (data not shown).

To further investigate the role of RPA, we monitored recombination induced by a double-strand break by Southern analysis. Mating type switching was induced in mutant and wild-type cultures by galactose induction of HO endonuclease from pJH727. After induction, DNA samples were extracted and analyzed by Southern blotting. Figure 3 shows efficient cutting at *MAT***a** by the HO endonuclease followed by the appearance of a product band (*MAT*α) in the wild-type control. The chromosomal *rfa1-t11* mutant generates a comparable level of HO-cleaved DNA but much less product DNA. Over time the HO-cleaved DNA in the *rfa1-t11* mutant disappeared, consistent with the cleaved DNA being degraded and the cells dying as was observed on transient exposure to galactose (discussed above). The *MAT*α product band produced in the *rfa1-t11* mutant represents 4.4% of the total *MAT***a** and α sequences, compared with 56% for the wild-type strain. The kinetics of appearance of *MAT*α product in the *rfa1-t11* mutant strain are indistinguishable from the wild-type strain. This defect in switching in the *rfa1-t11* mutant was confirmed by examination of the colonies produced after 5 hr of HO-induction. In the *rfa1-t11* mutant only 7% of the colonies maintaining the *GAL::HO* plasmid successfully switched from *MAT***a** to *MAT*α (55% switched in the wild-type strain). The *rfa1-t11* allele is not a null allele and retains some types of *RFA1* activity. Nevertheless, both the Southern blot analysis and the colony assays indicate that the *rfa1-t11* mutant is highly defective in its ability to carry out double-strand break-induced recombination.

Induction of HO endonuclease had a less clear effect on strains carrying the *rfa1-t49*, *rfa1-m51*, *rfa1-t23*, and *rfa1-t83* alleles (Figure 2), consistent with the observation that these alleles were among the moderate UV^S and MMS^S alleles identified. This suggests that these mutations only cause minor defects in homologous recombination, resulting in only moderate sensitivity to UV-irradiation, MMS, and HO endonuclease. Alternately, these mutations may not affect homologous recombination and may only cause defects in repair pathways like nucleotide excision repair or base excision repair.

Defects in single-strand annealing in the *rfa1-t11* mutant: Single-strand annealing (SSA) is a process in which single-stranded DNA regions are generated on each side of a double-strand break in the form of 3′ tails (Sugawara and Haber 1992). When complementary sequences on each side of the break become single stranded, they can anneal and subsequently be processed to result in a single copy, while deleting the intervening sequence. Given *RFA1*'s involvement in mating type switching, a process mediated by double-strand break repair, we tested whether *RFA1* has a role in SSA by utilizing an HO cut site situated between direct repeats of a 0.24-kb *E. coli lacZ* sequence carried on a centromeric plasmid. A Southern hybridization analysis of DNA extracted from a culture undergoing SSA revealed that the *rfa1-t11* mutant was impaired in its ability to carry out SSA relative to a wild-type strain (Figure 4). Although a small amount of product could be formed, the mutant was 8.5 times less efficient than wild type in carrying out SSA based on a densitometric analysis of the blots. This defect was also manifested by the loss of HO endonuclease-cleaved plasmid substrate in the mutant strain. The number of colonies retaining the plasmid after induction compared to those before

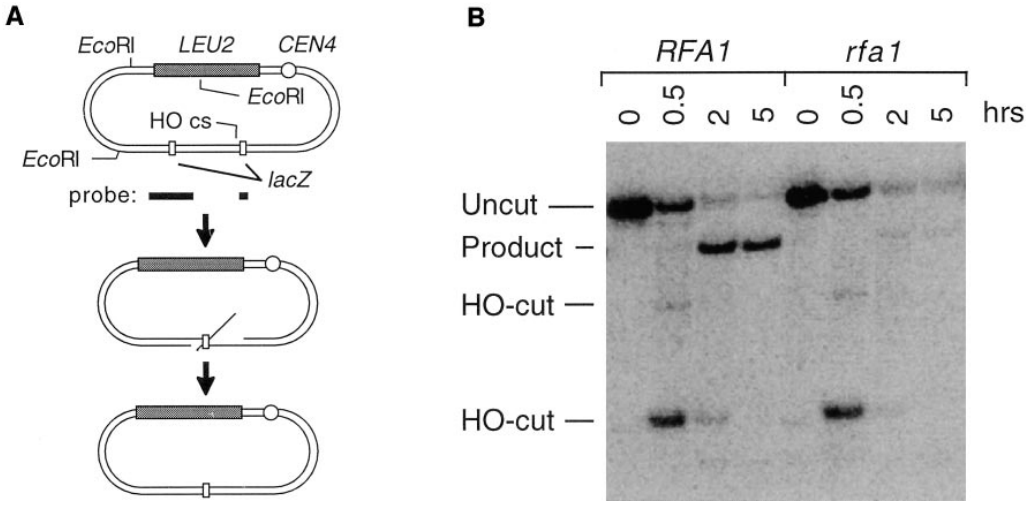


Figure 4.—*RFA1* is required for efficient single-strand annealing. (A) The plasmid pNSU208 (15 kb) contains two 0.24-kb direct repeats composed of *lacZ* sequences with an HO cut site in between. Cleavage by HO endonuclease is followed by 5′ to 3′ degradation of DNA leaving a 3′ single-stranded tail. The complementary *lacZ* sequences can anneal and be processed to yield a deletion product. (B) Single-strand annealing was initiated *in vivo* when a double-strand break was created by induction of the *GAL::HO* endonuclease gene in an *rfa1-t11* mutant containing the *rfa1* allele at the chromosomal locus and a wild-type strain. Cleavage of the 9.4-kb pNSU208 *Eco*RI fragment by HO endonuclease resulted in the production of 6.2- and 3.2-kb fragments by 0.5 hr and yielded a 7.8-kb product fragment in the wild-type strain. The amount of product formed was greatly reduced in the *rfa1-t11* mutant.

induction was 0.97 for wild type but only 0.12 for the *rfa1-t11* mutant.

Analysis of ts *rfa1* mutants: We identified 5 ts mutants (of 10 mutants that were retested) with which it was possible to demonstrate that the ts phenotype was associated with an *rfa1* mutation. Two of these mutant alleles (*rfa1-t6* and *rfa1-t33*) were successfully transferred to the chromosomal *RFA1* locus, whereas it was not possible to transfer the other three mutant alleles to the chromosomal *RFA1* locus (Figure 5). As shown in Figure 6, strains carrying the *rfa1-t33* and *rfa1-t6* alleles at either the chromosomal locus or on an *ARS CEN* plasmid had a clear temperature-sensitive phenotype. Strains carrying the *rfa1-t33* allele, both when integrated on the chromosome or on the *ARS CEN* plasmid, grew normally at the permissive temperature, while at the restrictive temperature they grew only at heavily streaked regions and formed no visible individual colonies. Strains carrying the chromosomal *rfa1-t6* allele grew more slowly at the permissive temperature than strains carrying this allele on an *ARS CEN* plasmid. Strains carrying the chromosomal *rfa1-t6* allele did not grow at all at the restrictive temperature, whereas strains carrying this allele on an *ARS CEN* plasmid grew only at heavily streaked regions

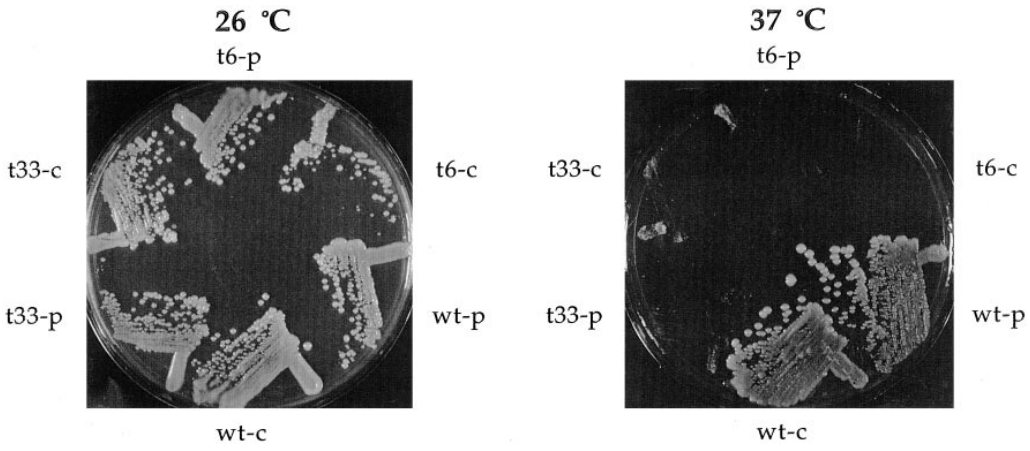


Figure 5.—Some *rfa1* mutants display a strong temperature-sensitive phenotype. The indicated mutant and wild-type strains were streaked on two YPD plates that were incubated at 26° (left panel) and at 37° (right panel) for 3 days, respectively. The allele number is indicated next to each streak. When the allele designation is followed by -c, it indicates the allele at the chromosomal locus, whereas -p indicates the allele carried on an *ARS CEN* plasmid. For wild-type control strains, RKY1900 was used as wt-p and W303-1A was used as wt-c.

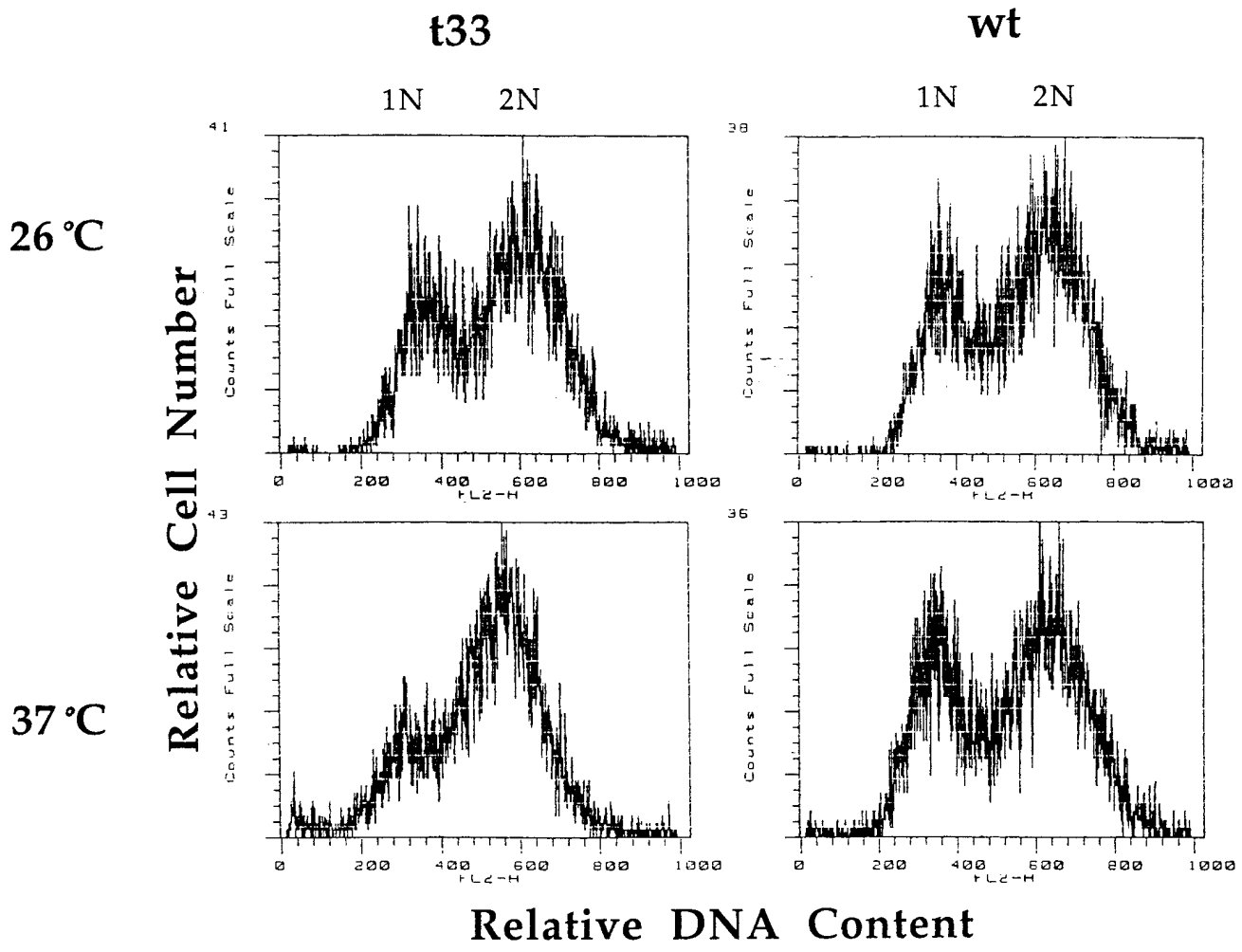


Figure 6.—FACS analysis of DNA content. Cells were grown in liquid YPAD to early logarithmic phase at 26°, and then the culture was divided into two portions: one portion was incubated at 26° and the other portion was incubated at 37°. Cells were withdrawn after 4 hr, stained with propidium iodide, and analyzed using a FACScan flow cytometer. The results obtained with the *rfa1-t33* allele integrated at the chromosomal locus and the wild-type strain W303-1A are shown here. Identical results were obtained when similar analysis was performed with the *rfa1-t6* allele at the chromosomal location.

and formed tiny colonies at the restrictive temperature. Thus, the ts phenotype caused by the integrated alleles (particularly in the case of the *rfa1-t6* allele) was more severe than the plasmid-born alleles. This suggests that the reason we were not able to transfer three of the ts *rfa1* alleles to the chromosomal locus was that they were lethal when integrated at the *RFA1* chromosomal locus.

The ts mutants did not initially appear to be sensitive to killing by UV-irradiation or MMS when they were characterized using quantitative assays as plasmid-born alleles. However, when the *rfa1-t33* and *rfa1-t6* alleles were transferred to the chromosomal locus, the resulting strains became sensitive to UV-irradiation and MMS. When these alleles were present on an *ARS CEN* plasmid, they conferred only limited sensitivity to UV-irradiation and MMS: Survival of a strain carrying a plasmid-born *rfa1-t33* allele was 0.143 and 0.116 after treatment with 75 J/m^2 of UV-irradiation and 0.5% MMS, respectively, and survival of a strain carrying a plasmid-born *rfa1-t6* allele was 8.27×10^{-2} and 8.4×10^{-2}, respectively, which was similar to that observed for the wild-type control strain (Figure 8). However, when these alleles were transferred to the chromosomal *RFA1* locus, they caused significantly increased sensitivity to both UV-irradiation and MMS at the standard doses tested: Survival of the *rfa1-t33* strain after treatment with 75 J/m^2 of UV-irradiation and 0.5% MMS was 1.01×10^{-2} and 1.83×10^{-3}, respectively, and survival of the *rfa1-t6* strain was 3.48×10^{-3} and 2.05×10^{-3}, respectively. These results suggest that expression of RPA1 from the chromosome is somewhat lower than that on an *ARS CEN* plasmid and that repair and/or recombination is more sensitive to RPA1 levels than DNA replication in the case of the ts alleles *rfa1-t6* and *rfa1-t33*, as compared to the repair-defective alleles *rfa1-t11* and *rfa1-t49* where no difference between plasmid and chromosomal alleles was observed (see above).

To characterize the ts phenotype conferred by the *rfa1-t33* and *rfa1-t6* alleles in more detail, we grew cultures of strains carrying these alleles at the permissive

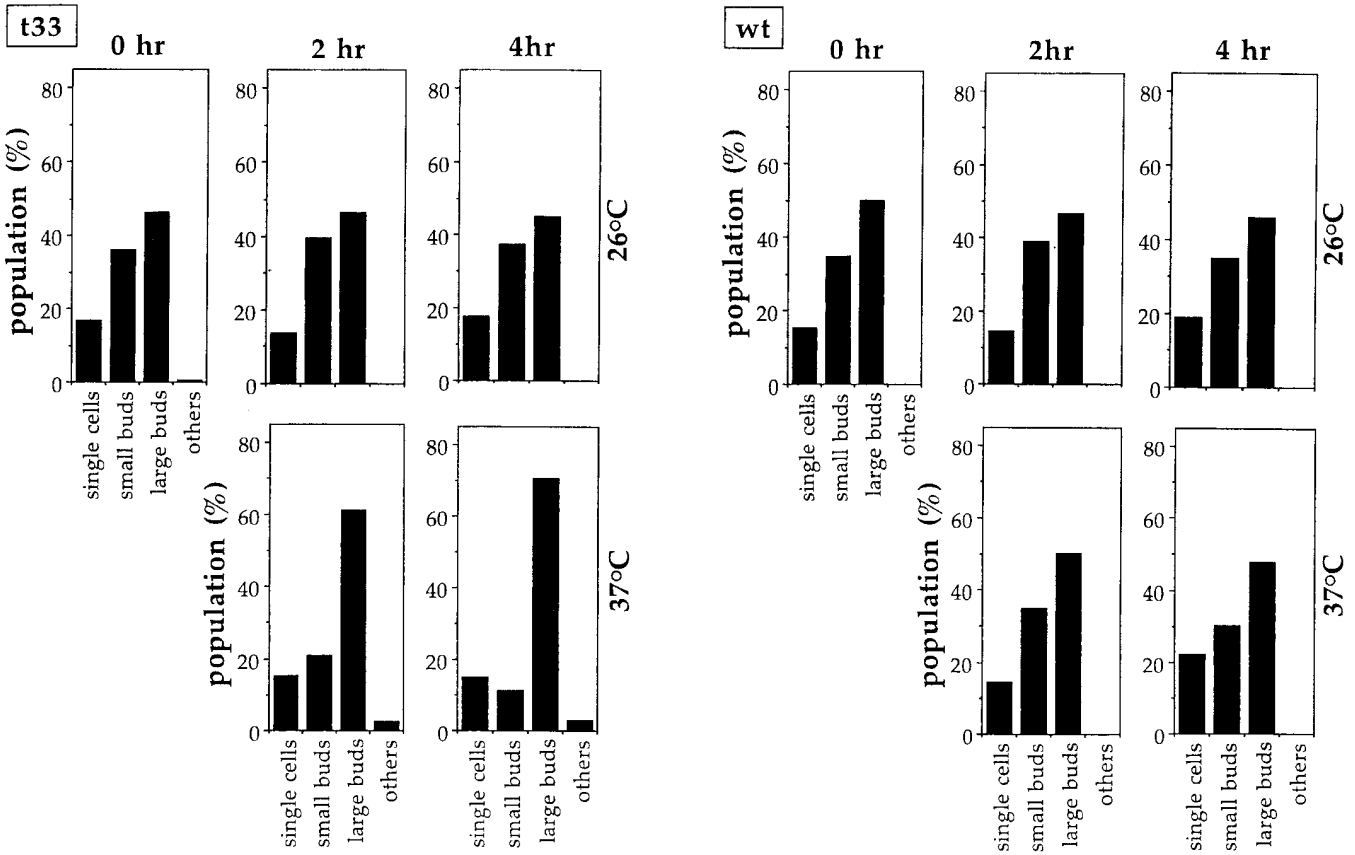


Figure 7.—Morphological analysis of wild-type and *rfa1-t33* cells at the restrictive temperature. A portion of the culture used in the experiments shown here was withdrawn at each indicated time point and examined by light microscopy. Cells in which the bud size was less than one-third the size of the mother cell were classified as "small buds," while the cells harboring larger buds were classified as "large buds." Cells having multiple buds or protruded buds were classified as "other." The results obtained with the *rfa1-t33* allele integrated at the chromosomal locus and the wild-type strain W303-1A are shown here. Identical results were obtained when similar analysis was performed with the *rfa1-t6* allele at the chromosomal location.

temperature, shifted logarithmically growing cultures to the nonpermissive temperature, and examined the resulting changes in cellular morphology and DNA content (Figures 6 and 7). The experiments presented were performed with a wild-type control strain and a strain carrying the *rfa1-t33* allele at the chromosomal locus; however, identical results were obtained with both the *rfa1-t33* and *rfa1-t6* alleles, regardless of whether they were on an *ARS CEN* plasmid or at the chromosomal *RFA1* locus (data not shown).

Figure 6 shows FACS analysis of the DNA content of wild-type and *rfa1-t33* cells (identical data were obtained with the *rfa1-t6* allele but are not presented due to space considerations). After 4 hr at the restrictive temperature, the *rfa1-t33* strain showed a clear difference in the distribution of cells having differing DNA contents compared to the wild-type strain. The *rfa1-t33* strain showed a significant decrease in the proportion of cells having either a 1N or 2N DNA content and a significant increase of the proportion of cells having greater than 1N but less than 2N DNA content compared to the wild-type control strain. This effect was seen to a lesser extent after 2 hr of incubation at the nonpermissive temperature and did not increase with continued incubation beyond 4 hr (data not shown). These results suggest that most of the *rfa1-t33* cells did not complete DNA replication and accumulated prior to M phase at the restrictive temperature. This was confirmed by analyzing the cells by light microscopy (Figure 7, discussed below); identical data were obtained with the *rfa1-t6* allele but are not presented due to space considerations. After 4 hr at the restrictive temperature, 70% of the *rfa1-t33* cells had arrested with a single large bud. After staining with DAPI, these large-budded cells were observed to have a single nucleus at the neck of the bud (data not shown). The large-budded cells appeared to have been derived from the small-budded cells presented in the culture at the time of temperature shift because the proportion of small-budded cells decreased by the same amount that the proportion of large-budded cells increased, whereas the proportion of nonbudded cells present in the culture did not appear to change. In addition, approximately 3% of the arrested *rfa1-t33* cells had protruded buds or dumbbell-shaped buds like those

observed after sporulation of *rfa1* null mutants (Heyer *et al.* 1990). Control experiments with the wild-type parental strain showed a normal distribution of cells at different stages of the cell cycle at both temperatures. From these results, we conclude that the ts *rfa1* mutants *rfa1-t33* and *rfa1-t6* have defects in the elongation stage of DNA replication at the restrictive temperature, resulting in cell cycle arrest at a premitotic stage, or they arrest prior to M phase at a step that causes a block in DNA replication.

In the experiments presented in Figures 6 and 7, we observed that prior to shifting to the restrictive temperature, 15–20% of the *rfa1-t33* cells had 1N DNA content and a similar proportion of the cells did not have buds consistent with 15–20% of the cells in G1. After 2 and 4 hr at the restrictive temperature, the proportion of such *rfa1-t33* cells did not change. This suggests that either these cells are dead or that they are unable to enter S phase and initiate DNA replication. Similar results were obtained with *rfa1-t6* cells (data not shown).

DNA sequence analysis of *rfa1* mutations: To determine the nature of the *rfa1* mutations causing the observed altered phenotypes, we sequenced the *rfa1* gene of all 19 UV^S and MMS^S alleles and both ts alleles. Figures 8 and 9 summarize the mutations identified and the predicted amino acid changes they cause. Every allele had at least one mutation in the *RFA1* gene. Thirteen alleles had either a single nucleotide change or two changes of which one was a silent change, allowing clear identification of the causal mutation. Eight alleles had two potentially significant nucleotide changes. There were no identical alleles; however, *rfa1-t155* could easily be related to *rfa1-t21* by the subsequent accumulation of a second, silent nucleotide change.

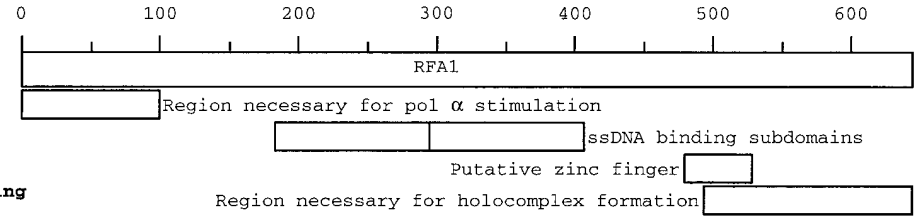
Twenty-three mutations were identified, and most of them changed amino acids that were conserved among yeast, human, *Drosophila melanogaster*, and *Xenopus lavis* (see Figure 8 for an analysis of the mutations, including a list of mutations affecting conserved amino acids). It is possible to ascribe significance to some of these mutations based on what is known about the structure of RFA1. Five mutations mapped in the region necessary for pol α stimulation by RFA (Kim *et al.* 1996). Three mutations causing very weak phenotypes mapped in the region between the regions required for pol α stimulation and DNA binding. Ten mutations, including two causing a high degree of sensitivity to UV-irradiation and MMS (*rfa1-t48* and *rfa1-m51*), mapped in either domain 1 or domain 2 of the conserved region of RFA1 required for ssDNA binding (Heyer *et al.* 1990; Gomes and Wold 1995; Philipova *et al.* 1996; Bochkarev *et al.* 1997). The mutations *rfa1-t21*, *rfa1-t155*, and *rfa1-t141* altered amino acids in the putative zinc finger of RFA1, which is not necessary for DNA-binding activity (Gomes and Wold 1995; Lin *et al.* 1996) but could act to mediate protein-protein interactions (Galcheva-Gargova *et al.* 1996; Rodgers *et al.* 1996; Shepard *et al.* 1996; Szabo *et al.* 1996). Finally, at least one mutation mapped in the C-terminal region required for RPA holocomplex formation. Understanding the exact effects of each mutation on the function of RPA1 will require a detailed analysis of the biochemical properties of the mutant RPA1 proteins. However, these data do allow us to make some predictions about the functions of the different regions of RPA1, and these will be discussed below.

In the case of alleles having two nucleotide changes, it is not clear which nucleotide change is the causal mutation. However, some insights into the nature of these alleles can be obtained by comparing the properties of these alleles. The *rfa1-t22* allele had two nucleotide changes, one of which in position 43 was in common with the *rfa1-t47* allele that had only that change. Because the *rfa1-t47* allele causes a lower degree of UV- and MMS-sensitivity than the *rfa1-t22* allele, it seems likely that the second nucleotide change present in the *rfa1-t22* allele (position 146) also contributes to the mutant phenotype caused by the *rfa1-t22* allele. The *rfa1-t11* and *rfa1-t69* alleles cause a similar degree of UV- and MMS-sensitivity. This suggests that the common nucleotide change at position 133 might be the causal mutation, while the second nucleotide change in the *rfa1-t69* allele at position 362 may not contribute to the mutant phenotype. One of the strong ts alleles, *rfa1-t6*, shared the same nucleotide change at position 1051 with the *rfa1-t83* allele, which had an additional change at position 1805. Because the *rfa1-t83* allele caused moderate UV- and MMS-sensitivity in addition to a ts phenotype, the latter change at position 1805 might be responsible for its UV- and MMS-sensitive phenotypes, while the former change at position 1805 might be the causal mutation for temperature sensitivity. It will be necessary, however, to use site-directed mutagenesis to construct appropriate single mutants to fully understand the nature of the alleles containing more than one potentially significant nucleotide change.

The *rfa1-t33* and *rfa1-t92* alleles had one nucleotide change in common, a T to C change at position 1117 that changed the serine to a proline. The *rfa1-t33* allele caused a strong ts phenotype, whereas the *rfa1-t92* allele just showed slight UV-sensitivity (Figure 8). These results suggest that the second mutation in *rfa1-t92* suppressed the ts phenotype caused by the serine to proline change and caused the weak UV-sensitive phenotype, possibly in combination with the serine to proline change.

DISCUSSION

In an effort to understand the role of RPA in DNA metabolism and to begin to identify regions of RPA required for different RPA-dependent processes, we have used a plasmid shuffle method to isolate random mutations in the *RFA1* gene encoding the 70 kDa sub-



Allele Name	UV^S	MMS^S	TS	HO^S	Doubling Time	
t49	30	121	Y	s	130	(Ile->Ser)$_{14}$
t47	5	4	N	nt	100	(Phe->Leu)$_{15}$
t22	100	607	N	ss	165	(Phe->Leu)$_{15}$ (Met->Thr)$_{49}$
t11	221	2429	N	ss	120	(Lys->Glu)$_{45}$
t69	200	3470	N	ss	130	(Lys->Glu)$_{45}$ (Asp->Gly)$_{121}$
t26	4	7	N	nt	100	(Arg->Cys)$_{109}$
t73	4	2	N	nt	100	(Phe->Ser)$_{124}$ (Glu->Val)$_{562}$
t92	2	1	N	nt	100	(Phe->Ser)$_{124}$ (Ser->Pro)$_{373}$
t124	5	3	N	nt	110	(Gly->Arg)$_{207}$ (Tyr->Cys)$_{255}$
t48	247	2833	N	ss	165	(Leu->Pro)$_{221}$
t23	9	11	N	-	135	(Phe->Ser)$_{222}$ (His->Arg)$_{274}$
m51	41	179	N	s	140	(Ser->Pro)$_{230}$
t6	2	4	Y	nt	nt	(Ser->Pro)$_{351}$
t83	7	10	Y	s	120	(Ser->Pro)$_{351}$ (Leu->Ser)$_{602}$
t33	2	3	Y	nt	nt	(Ser->Pro)$_{373}$
t63	3	3	N	nt	100	(Ser->Pro)$_{391}$
t19	2	4	Y	nt	100	(Leu->Ser)$_{410}$ (Ser->Cys)$_{569}$
t141	3	3	N	nt	110	(Lys->Glu)$_{493}$
t21	36	136	N	sss	130	(Lys->Glu)$_{494}$
t155	33	472	N	sss	140	(Lys->Glu)$_{494}$
t18	10	28	N	sss	110	(Phe->Ile)$_{537}$

Figure 8.—Summary of the phenotypes caused by *rfa1* mutations and illustration of the positions of the mutations relative to the positions of known RFA1 structural features. The coordinates of the different functional regions are taken from the papers discussed in the introduction and discussion (Erdile *et al.* 1991; Adachi and Laemmli 1992; Brown *et al.* 1994; Gomes and Wold 1995, 1996; Ishiai *et al.* 1996; Kim *et al.* 1996; Lin *et al.* 1996; Park *et al.* 1996; Philipova *et al.* 1996; Bochkarev *et al.* 1997). The positions of the indicated amino acid changes are given relative to the structural features of the protein and are based on the following mutational data: Ile14Ser, T41G; Phe15Leu, T43C; Met49Thr, T146C; Lys45Glu, A133G; Asp121Gly, A362G; Arg109Cys, C325T; Phe124Ser, T371C; Glu562Val, A1685T; Ser373Pro, T1117C; Gly207Arg, G619A; Tyr255Cys, A764G; Leu221Pro, T662C; Phe222Ser, T665C; His274Arg, A821G; Ser230Pro, T688C; Ser351Pro, T1051C; Leu602Ser, T1805C; Ser391-Pro, T1171C; Leu410Ser, T1229C; Ser569Cys, A1705T; Lys493Glu, A1477G; Lys494Glu, A1480G; Phe537Ile, T1609A. The mutational changes are annotated in the following manner: open triangle, residue mutated is not conserved among *S. cerevisiae*, human, Drosophila, and Xenopus RPA1; closed triangle, residue mutated is conserved among *S. cerevisiae*, human, Drosophila, and Xenopus RPA1. In addition, the following silent nucleotide changes were found: *rfa1-t22*, A255T; *rfa1-t69*, T195C; *rfa1-t23*, A630G; *rfa1-t63*, C1074G; *rfa1-t155*, T1533C. UV- and MMS-sensitivity were determined at a fixed dose of either 75 J/m^2 of UV-irradiation or 40 min of incubation in 0.5% MMS as described in materials and methods and in Figure 1: the values given are the fold reduction in survival relative to the wild-type strain. (The fraction of wild-type cells that survived UV treatment was 0.21 and the fraction of wild-type cells that survived MMS treatment was 0.34.) HO sensitivity summarizes the data presented in Figure 2: —, average of >20 colonies per patch survive upon HO induction (wild-type level); s, average of 10–20 colonies per patch survive upon HO induction; ss, average of 6–10 colonies per patch survive upon HO induction; sss, average of 0–5 colonies per patch survive upon HO induction; nt, not tested. Temperature sensitivity refers to whether a consistent temperature-sensitive phenotype like that illustrated in Figure 5 was seen as defined in materials and methods under *Semiquantitative assays.* The doubling times presented were determined by growing the indicated strains in liquid YPD medium at 26° and monitoring cell growth both by counting cell numbers and by plating onto YPD plates to determine the number of viable cells present. All of the data summarized in this figure were generated with strains in which the *rfa1* alleles were present on a plasmid.

unit of RPA. These mutants were then screened for ts, UV^S, and MMS^S, and 24 mutants having a variety of different phenotypes were obtained. In addition, we have retained a large number of other uncharacterized *rfa1* mutants that will be useful for future studies. Previous studies described the first *rfa1* mutants and some of the phenotypes caused by *rfa1* mutations; however, these studies identified only a limited number of *rfa1* mutants (Heyer *et al.* 1990; Brill and Stillman 1991; Longhese *et al.* 1994; Firmenich *et al.* 1995; Smith and

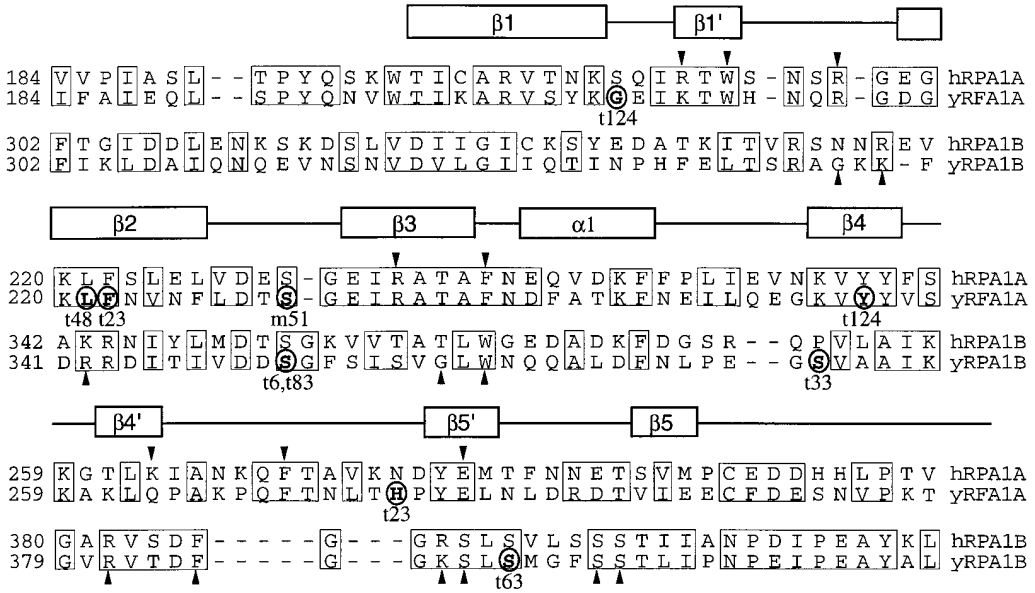


Figure 9.—Alignment of different RPA sequences and amino acid changes caused by *rfa1* mutations. Alignment of the predicted RFA1 amino acid sequences for the ssDNA binding domains 1 and 2 of *S. cerevisiae*, human, Drosophila and Xenopus RFA1 (see Philipova *et al.* 1996; Bochkarev *et al.* 1997). Identities are indicated by boxes, and the positions of the amino acid changes from Figure 8 are indicated by circles and are labeled with the relevant allele numbers. *S. cerevisiae* and human are indicated by y and h, respectively. Domains 1 and 2 are indicated as RPA1A and RPA1B, respectively. Down-arrows indicate ssDNA-binding contacts in domain 1 and up-arrows indicate ssDNA-binding contacts in domain 2.

Rothstein 1995). The preliminary analysis of the *rfa1* mutants described here has documented a variety of phenotypes caused by *rfa1* mutations, including several causing much stronger replication and recombination defects than previously described. Based on the results obtained thus far, it is likely that we will be able to use the mutant collection described here in conjunction with considerable additional experimentation to define in greater detail different roles of RPA in DNA metabolism.

Biochemical studies of RPA have shown that it stimulates the homologous pairing activity of several different homologous pairing proteins, SEP1 (Heyer *et al.* 1990; Alani *et al.* 1992), HPP1 (Moore *et al.* 1991), and RAD51 (Sung 1994, 1997; Sugiyama *et al.* 1997) and interacts with proteins required for excision repair (He *et al.* 1995; Lee *et al.* 1995; Li *et al.* 1995; Matsuda *et al.* 1995; Matsunaga *et al.* 1996) and genetic recombination (Firmenich *et al.* 1995; Park *et al.* 1996). Consistent with this, we were able to isolate a number of *rfa1* alleles that conferred different degrees of sensitivity to UV-irradiation and MMS (Figures 1 and 8). Interestingly, most of these alleles were not temperature-sensitive and did not appear to cause defects in DNA replication. Similarly, many of the mutations that caused replication defects had only limited effects on repair of UV- and MMS-induced damage to DNA. These results suggest that there are a number of ways in which mutations in *RFA1* may alter the properties of RPA without changing its ability to function in DNA replication. This may reflect the possibility that the DNA and protein-protein interaction requirements for RPA1 may differ significantly between DNA replication and DNA recombination/repair. For example, the ability of RPA1 to interact with and stimulate the activity of XPA, XPF, XPG, and RAD52 (Firmenich *et al.* 1995; He *et al.* 1995; Lee *et al.* 1995; Li *et al.* 1995; Matsuda *et al.* 1995; Matsunaga *et al.* 1996; Park *et al.* 1996) likely reflects a function that is required for recombination and repair but not for replication. An interesting parallel in this regard is the *E. coli* SSB, where the *ssb-113* mutation alters its DNA-binding properties so as to cause repair and recombination defects but not replication defects (Meyer and Laine 1990).

Previous genetic studies have provided evidence that RPA functions in genetic recombination (Longhese *et al.* 1994; Firmenich *et al.* 1995; Smith and Rothstein 1995) and interacts with at least one recombination protein. Two of these studies reported the isolation of *rfa1* alleles that caused modest, less-than-10-fold reductions in direct repeat recombination (Smith and Rothstein 1995) and mitotic intrachromosomal recombination (Longhese *et al.* 1994), respectively. One of these alleles also stimulated recombination between direct repeats in a *rad1 rad52* double mutant (Smith and Rothstein 1995). A third study reported the isolation of an *rfa1* allele (*rfa1-44*) that caused a strong defect in a double-strand break-induced plasmid by chromosome gene conversion (Firmenich *et al.* 1995). Subsequent studies have shown that the *rfa1-44* allele caused ~50%

failure in mating type switching (J. E. Haber and N. Sugawara, unpublished data), a result that is not surprising because the mutant isolation procedure used involved induction of HO endonuclease in a *MAT* strain that should be lethal in the presence of mutations causing strong defects in double-strand break repair. We found that many of the UV^S and MMS^S mutants described here showed some degree of sensitivity to killing by HO endonuclease compared to the wild-type control strain (Figure 2), suggesting they might be highly defective in recombinational repair of double-strand breaks. More detailed analysis of one UV^S and MMS^S mutant (*rfa1-t11*) using genetic and biophysical assays that measure mating type switching showed that this allele caused over a 90% decrease in mating type switching in physical assays (Figure 3), consistent with a strong defect in double-strand break repair. The *rfa1-t11* allele also caused ~90% decrease in SSA recombination; this is the first report of such an effect of an *RFA* allele. Interestingly, the *rfa1-t11* allele was not the most HO endonuclease-sensitive, UV^S, or MMS^S allele we obtained (Figures 2 and 8), suggesting that there are a number of mutants among our collection that are even more recombination defective than *rfa1-t11*. More detailed analysis of these mutants will be performed in the future to more completely determine their effects on different types of recombination.

Many of the *rfa1* mutants we isolated confer a UV^S phenotype. This is consistent with the view that RPA might play a direct role in the excision repair of UV damage. Such a role for RPA is based on the observations that human RPA is required for excision repair *in vitro* (Coverley *et al.* 1991) and that RPA1 interacts with proteins required for excision repair (Aboussekhra *et al.* 1995; He *et al.* 1995; Lee *et al.* 1995; Li *et al.* 1995; Matsuda *et al.* 1995; Matsunaga *et al.* 1996). However, the UV^S phenotypes we observed could be due to defects in either excision repair or recombinational repair of UV damage. Given the MMS-sensitivity and HO-endonuclease sensitivity of many of the UV^S mutants we obtained, it would not be surprising if the UV^S phenotype observed reflected a defect in recombinational repair rather than excision repair. Additional analysis will be required to distinguish between these possibilities.

RPA was originally identified because of its requirement for *in vitro* DNA replication, suggesting that it is an essential DNA replication factor (Wobbe *et al.* 1987; Wold *et al.* 1987; Fairman and Stillman 1988), and several genetic studies have provided limited data supporting this view. Analysis of the terminal arrest phenotype of spores containing an *rfa1* null allele showed that such spores germinate and arrest with either a large bud or multiple large buds and a single nucleus consistent with a lack of DNA replication (Heyer *et al.* 1990; Brill and Stillman 1991). In addition, *rfa1-M2* mutant cells were weakly temperature-sensitive and a higher proportion of cells held at the restrictive temperature had an S phase DNA content than wild-type cells suggestive of some defect in DNA replication (Longhese *et al.* 1994). In the studies presented here we identified two *rfa1* alleles (*rfa1-t6* and *rfa1-t33*) that caused a strong temperature-sensitive phenotype (Figure 5). In both cases, 70% of the mutant cells held at the restrictive temperature arrested with a large bud having a single nucleus at the neck of the bud (data not shown), and consistent with this, 80 to 85% of the cells held at the restrictive temperature had a sub-2N DNA content. [The data for the *rfa1-t33* allele are presented in Figures 6 and 7; however, identical results were obtained with the *rfa1-t6* allele (data not shown).] The large-budded cells appeared to have accumulated from small-budded cells (S phase cells) that progressed through the cell cycle and arrested prior to M phase because the proportion of small-budded cells decreased by the same amount as the proportion of large-budded cells increased during incubation at the restrictive temperature. In contrast, the proportion of nonbudded cells present in the culture did not appear to change during the time course of the experiment. These data are consistent with the idea that the *rfa1-t6* and *rfa1-t33* alleles cause a defect in the elongation stage of DNA replication resulting in the accumulation of cells prior to M phase. Alternately, these alleles could cause some other type of S-phase/G2-phase defect, in turn causing some type of inappropriate progression through the cell cycle resulting in a failure to complete DNA replication and arrest prior to M phase. In addition, 15 to 20% of the cells held at the restrictive temperature remained as single cells, and consistent with this, 15 to 20% of the cells held at the restrictive temperature had a 1N DNA content. Assuming that the nonbudded cells and the 1N DNA content cells are not simply dead cells, one interpretation of these latter results is that the *rfa1-t6* and *rfa1-t33* alleles also cause a defect in the initiation stage of DNA replication leading to some of the cells arresting in G1. These genetic properties of the *rfa1-t6* and *rfa1-33* alleles are consistent with the view that RPA1 is an essential DNA replication factor both in the initiation and the elongation stages (Tsurimoto and Stillman 1989; Kenny *et al.* 1990; Collins and Kelly 1991; Lee *et al.* 1991; Dornreiter *et al.* 1992).

A considerable amount of data is now available about the structure and function of different regions of RFA1, allowing us to relate the phenotypes of the different *rfa1* mutations described here to the functional regions of RFA1 in which they map (Figures 8 and 9). Five mutations, including the *rfa1-t11* allele that caused strong defects in HO-induced double-strand break repair and SSA recombination, mapped in the region of RFA1 required for pol α stimulation (Kim *et al.* 1996), a region whose *in vivo* function is not known. These mutants generally have strong UV^S, MMS^S, and HO^S phenotypes, indicating this region is important for recombination and repair. One of these mutants was also

weakly ts, suggesting this region might not be very important for DNA replication. Three mutations mapped in the region between the regions required for pol α stimulation and DNA binding, and all of these caused very weak phenotypes suggesting this region is also of limited functional significance or is tolerant of the amino acid changes observed. These results are consistent with the observation that this region of the protein is not required for SV40 replication *in vitro* (Gomes and Wold 1996). Mutations were found in both domains 1 and 2 of the ssDNA-binding region (Gomes and Wold 1995; Kim *et al.* 1996; Lin *et al.* 1996; Bochkarev *et al.* 1997). Mutations in the N-terminal half of each of the two domains caused strong UV^S and MMS^S phenotypes, whereas mutations in the C-terminal half of domain 2 only caused weak UV^S and MMS^S phenotypes. Interestingly, none of the mutations in domain 1 caused a ts phenotype, whereas many of the domain 2 mutations caused a ts phenotype. Of particular significance in this regard were mutant alleles *rfa1-m51* and *rfa1-t6*, which each caused a Ser-to-Pro change at the equivalent amino acid in domains 1 and 2, respectively; yet *rfa1-m51* caused a strong UV^S, MMS^S phenotype whereas *rfa1-t6* caused a strong ts phenotype in combination with a weak UV^S, MMS^S phenotype that was considerably weaker than the UV^S, MMS^S phenotype caused by the *rfa1-m51* mutation. These data suggest that domains 1 and 2 do not play equivalent roles in RFA1. In some regards, mutations in domain 1 are reminiscent of the *E. coli ssb113* mutation that alters the DNA-binding properties on SSB in a way that affects recombination and repair but not DNA replication (Meyer and Laine 1990). Three mutations were found in the Zn finger domain that is in close proximity to the region required for RFA holocomplex formation, and therefore interaction between RFA1 and RAD52 via RFA2 while an additional mutation mapped in the holocomplex formation region (Erdile *et al.* 1991; Adachi and Laemmli 1992; Brown *et al.* 1994; Gomes and Wold 1996; Ishiai *et al.* 1996; Lin *et al.* 1996; Lin *et al.* 1997). Interestingly, three of these mutations caused the greatest sensitivity to HO endonuclease-induced double-strand breaks. These mutations caused strong UV^S, MMS^S, and HO^S phenotypes but did not cause a ts phenotype. It is possible that these phenotypes result from an altered ability of mutant RFA1 proteins to recruit proteins like RAD52 required for recombination and repair (Park *et al.* 1996) because of alterations in holocomplex structure and/or RFA1-RFA2 interactions. The detailed biochemical analysis of these mutant proteins may prove insightful in understanding the biochemical functions of RPA.

In summary, we have described the isolation of 24 *rfa1* mutants having a variety of different ts, UV^S, and MMS^S phenotypes. The analysis of these mutants has yielded at least two significant results not previously obtained in studies of *rfa1* mutants. First, we have described the first strong double-strand break repair-defective and SSA-defective *rfa1* mutant, providing strong evidence for a role of RPA in different types of double-strand break repair. Second, we have described a variety of ts lethal *rfa1* mutations, and our data suggest that at least two of these mutations confer cell cycle progression defects consistent with RPA having roles in both the initiation and elongation stages of DNA replication. Third, we have provided genetic evidence that the replicative and recombination/repair properties of RPA may be separable. Continued analysis of the mutant collection described here is likely to provide insight into other roles that RPA may have in DNA metabolism.

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