Roles of Replication Protein-A Subunits 2 and 3 in DNA Replication Fork Movement in *Saccharomyces cerevisiae*

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ABSTRACT

Replication Protein-A, the eukaryotic SSB, consists of a large subunit (RPA1) with strong ssDNA binding activity and two smaller subunits (RPA2 and 3) that may cooperate with RPA1 to bind ssDNA in a higher-order mode. To determine the *in vivo* function of the two smaller subunits and the potential role of higher-order ssDNA binding, we isolated an assortment of heat-lethal mutations in the genes encoding RPA2 and RPA3. At the permissive temperature, the mutants show a range of effects on DNA replication fidelity and sensitivities to UV and MMS. At the nonpermissive temperature, four out of five RPA2 mutants show a fast-stop DNA synthesis phenotype typical of a replication fork block. In contrast, the fifth RPA2 mutant and all RPA3 mutants are able to complete at least one round of DNA replication at the nonpermissive temperature. The effect of these mutations on the stability of the RPA complex was tested using a coprecipitation assay. At the nonpermissive temperature, we find that RPA1 and RPA2 are dissociated in the fast-stop mutants, but not in the slow-stop mutants. Thus, replication fork movement *in vivo* requires the association of at least two subunits of RPA. This result is consistent with the hypothesis that RPA functions *in vivo* by binding ssDNA in a higher-order mode.

REPPLICATION Protein-A (RPA) is a three-subunit single-stranded DNA binding protein that was originally identified as a human protein required for SV40 DNA replication *in vitro* (WOBBE *et al.* 1987; FAIRMAN and STILLMAN 1988; WOLD and KELLY 1988). Human RPA (hsRPA) is composed of 70-, 34-, and 11-kDa subunits with a potent ssDNA binding activity and a low affinity for dsDNA and RNA (KIM *et al.* 1992). In *Saccharomyces cerevisiae* scRPA is composed of 69- (RPA1; encoded by *RFA1*), 36- (RPA2; encoded by *RFA2*), and 13-kDa (RPA3; encoded by *RFA3*) subunits and each subunit is essential for viability (BRILL and STILLMAN 1989, 1991; HEYER *et al.* 1990).

RPA is likely to be the eukaryotic homologue of the homotetrameric SSB of *Escherichia coli* (EcSSB). Like EcSSB, RPA is not only required for DNA replication *in vitro*, but is required for DNA repair (COVERLEY *et al.* 1991, 1992; GUZDER *et al.* 1995; HE *et al.* 1995; LEE *et al.* 1995; LI *et al.* 1995; MATSUDA *et al.* 1995) and recombination (HEYER and KOLODNER 1989; MOORE *et al.* 1991; FIRMENICH *et al.* 1995; SMITH and ROTHSTEIN 1995). Only the function of the large subunit (RPA1) has been clearly defined, however. RPA1 has been shown to bind ssDNA on its own (BRILL and STILLMAN 1989; WOLD *et al.* 1989; KENNY *et al.* 1990; ERDILE *et al.* 1991), and truncation analysis revealed that the ssDNA binding domain of hsRPA1 lies within the first 441 resi-

dues of the protein (GOMES and WOLD 1995). In yeast, RPA1 was shown to contain two tandem copies of a 120-amino acid domain with ssDNA binding activity. This 120-amino acid region has weak sequence similarity to an EcSSB protomer and was termed an ssDNA binding domain, or SBD (PHILIPPOVA *et al.* 1996).

The functions of the two smaller subunits of RPA are unknown. Neither subunit shows strong ssDNA binding activity, but both subunits are required for RPA function *in vitro*. For example, the p70 subunit of hsRPA alone is unable to support SV40 DNA replication, and antibodies to p34 inhibit the reaction (KENNY *et al.* 1990; ERDILE *et al.* 1991). Further, hsRPA containing a C-terminal truncation of p34 shows reduced activity in SV40 DNA replication (LEE and KIM 1995). Several observations suggest a regulatory role for the p34 subunit. The p34 subunit is subject to cell-cycle-dependent phosphorylation (DIN *et al.* 1990; DUTTA and STILLMAN 1992; FOTEDAR and ROBERTS 1992) and immunolocalization (CARDOSO *et al.* 1993; MURTI *et al.* 1996) as well as an interaction with the DNA repair protein XPA (LEE *et al.* 1995; LI *et al.* 1995; MATSUDA *et al.* 1995) and *in vitro* phosphorylation by DNA-dependent protein kinase (BRUSH *et al.* 1994). Some of these studies indicate that p34 phosphorylation is not essential for SV40 DNA replication or nucleotide excision repair (BRUSH *et al.* 1994; HENRICKSEN and WOLD 1994; LEE and KIM 1995; PAN *et al.* 1995), but is required to reverse the inhibitory effect of DNA-dependent protein kinase (HENRICKSEN *et al.* 1996).

We have recently suggested that the minimal essential

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regions of yeast RPA subunits 2 and 3 have sequence similarity to the SBDs of RPA1 and that, like the homotetrameric Ecssb, the RPA heterotrimer is composed of four SBDs (PHILIPOVA *et al.* 1996). RPA, like Ecssb, may also have multiple ssDNA binding modes. In the case of Ecssb, two protomers bind 35 nucleotides (nt) of ssDNA at low salt concentrations while four protomers bind 65–90 nt at higher ionic strength (LOHMAN and OVERMAN 1985; LOHMAN and FERRARI 1994; RIGLER and ROMANO 1995). This binding mode causes compaction of the ssDNA due to higher-order binding or “wrapping” about the tetramer (CHRYSOGELOS and GRIFFITH 1982; BUJALOWSKI *et al.* 1988). In the case of scRPA, we have shown that RPA2 can bind ssDNA as part of the RPA trimer in a salt-dependent reaction similar to the higher-order wrapping of ssDNA about Ecssb (PHILIPOVA *et al.* 1996). Wrapping of ssDNA has also been observed by electron microscopy (EM) of scRPA bound to ssDNA (ALANI *et al.* 1992). While EM studies of hsRPA bound to ssDNA have also shown a salt-dependent compaction of ssDNA, these investigators suggest that compaction results from structural changes other than wrapping of ssDNA about hsRPA (TREUNER *et al.* 1996). While the biological significance of this mode of ssDNA binding is unclear, its conservation between such different organisms suggests that it is likely to be involved in some significant aspect of cellular DNA metabolism.

To investigate the *in vivo* function of RPA2 and RPA3, we isolated a number of temperature-sensitive (T_s^-) mutations in the genes for these subunits. Here we characterize these alleles and show that they result in a range of phenotypes in DNA replication and repair. At the nonpermissive temperature most, but not all, RPA2 mutants accumulate in the G2 phase of the cell cycle while RPA3 mutants accumulate primarily in G1. Further, most RPA2 mutants show a quick-stop DNA synthesis phenotype while RPA3 mutants are able to complete one round of DNA replication at the nonpermissive temperature. We demonstrate a strict correlation between the quick-stop phenotype and dissolution of the RPA1/RPA2 complex. Thus, RPA2 is likely to play a direct role in replication fork movement. Based on our current model of RPA, we conclude that higher-order ssDNA binding by RPA2 is essential for replication fork movement.

MATERIALS AND METHODS

Isolation of RPA2 and RPA3 mutants: Conditional-lethal mutations in the genes encoding RPA2 and RPA3 were isolated using three approaches. First, a systematic truncation analysis of *RFA2* and *RFA3* indicated that the largest viable truncations of these genes conferred growth at 30°, but not at 37° (PHILIPOVA *et al.* 1996). Second, clusters of charged amino acids within RPA2 were changed to alanine by site-directed mutagenesis (KUNKEL 1985) and screened for temperature-sensitive growth as described (WERTMAN *et al.* 1992). The template for this mutagenesis, pJM229, contains the wild-type *RFA2* cDNA and promoter on a *Pst*I/*Xmn*I fragment

(BRILL and STILLMAN 1991) in pRS413 (SIKORSKI and HIETER 1989). The following 14 individual coding changes were made via mutations in *RFA2*: H67A/H68A, D80A/H81A, E90A/D91A, R99K/K100A, E103A/D104A, D113A/D114A, K141A/E142A, K146A/K147A, H166A/H167A, K199A/D200A/D201A, K219A/K220A, K225A/K226A, D244A/E245A, D266A/D267A. These 14 mutant *rfa2* plasmids were introduced into strain SBY105 (Table 1) by selecting for histidine prototrophy. This yeast strain carries a chromosomal deletion of *RFA2* and a wild-type copy of *RFA2* in the centromeric *URA3* plasmid, YCp50 (Table 2) (PHILIPOVA *et al.* 1996). Transformants were streaked onto plates containing 5-fluoroorotic acid (FOA) (BOEKE *et al.* 1987) and tested for growth at 25° and 37°. Only the E90A/D91A mutant failed to grow at 37°. Finally, random mutagenesis of *RFA2* and *RFA3* was performed by mutagenic PCR essentially as described (LEUNG *et al.* 1989). Plasmids pJM215 and pJM318 (BRILL and STILLMAN 1991) were individually subjected to 35 cycles of amplification using Taq DNA polymerase in the presence of 1 mM $MnCl_2$ and the universal forward and reverse sequencing primers. The PCR products were digested with *Bam*HI and *Sal*II, ligated into the yeast centromeric plasmid pRS415, and transformed into *E. coli*. The libraries (~100,000 clones each) of mutagenized *RFA2* and *RFA3* DNA were amplified in bacteria and introduced into yeast strains SBY105 and SBY111, respectively, by selecting for leucine prototrophy. SBY111 carries a chromosomal deletion of *RFA3* and a wild-type copy of the *RFA3* gene in YCp50 (PHILIPOVA *et al.* 1996). Leucine prototrophs (~50,000 each) were replica-plated onto media containing FOA and placed at 37°. Colonies that failed to grow at 37° were recovered from the original transformation plate and the plasmid isolated in *E. coli* and rechecked for temperature-sensitive growth in yeast.

All *rfa-ts* genes were then subcloned into the integrating vector pRS403. *RFA2* alleles were directed to integrate at the *HIS3* locus of SBY105 by linearizing the pRS403 derivatives with *Nde*I, or *Msc*I (*rfa2-C100* and *RFA2* wild-type). *RFA3* alleles were directed to integrate at the *HIS3* locus of SBY111 by linearizing the pRS403 derivatives with *Nhe*I. Stable T_s^- integrants were then obtained by selection on FOA. All strains were grown at the permissive temperature of 25° unless otherwise noted.

Survival curves: Strains were grown in liquid YPD medium to early log phase and shifted to the nonpermissive temperature (37°) in a shaking water bath. Aliquots of cells were taken at 0, 2, 4, 6, and 8 hr after the temperature shift and sonicated to disrupt cell clumps. Approximately 300 cells were then spread on 10-cm YPD plates and placed at 25°. The number of viable cells was determined by counting colonies after 3 days of growth.

UV, MMS, and α -aminoadipate sensitivity: To measure sensitivity to ultraviolet (UV) light, yeast strains were grown in YPD medium to early log phase and ~500 cells spread onto 10-cm YPD plates. The plates were then irradiated with the indicated levels of ultraviolet light using a UV crosslinker (Stratagene). The number of surviving cells was determined by counting colonies after 3 days growth at room temperature. Methyl-methanesulfonate (MMS) sensitivity was determined in liquid culture by adding MMS (Sigma) to a final concentration of 0.1% to exponentially growing cells in YPD. At the indicated times, aliquots were removed and neutralized with an equal volume of 10% sodium thiosulfate. The cells were then washed with water and an appropriate volume (initially containing 300 cells) spread onto YPD plates. The number of colonies was determined after 3 days growth at room temperature. Mutator analysis was carried out by growing seven independent cultures of each yeast strain to early log phase and plating 10^6 (or 10^7 for wild type) cells onto synthetic medium containing yeast nitrogen base without amino acids

TABLE 1

S. cerevisiae strains used in this study

Strain	Genotype ^a	Reference
W303-1a	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	THOMAS and ROTHSTEIN (1989)
SBY105	W303-1a <i>rfa2-1::TRP1</i> [pJM218 (<i>RFA2 URA3</i>)]	PHILIPOVA <i>et al.</i> (1996)
SBY111	W303-1a <i>rfa3-1::TRP1</i> [pJM320 (<i>RFA3 URA3</i>)]	PHILIPOVA <i>et al.</i> (1996)
HMY357	W303-1a <i>rfa2-1::TRP1 HIS3::RFA2^b</i>	This study
HMY344	W303-1a <i>rfa2-1::TRP1 HIS3::rfa2-55^b</i>	This study
HMY343	W303-1a <i>rfa2-1::TRP1 HIS3::rfa2-210^b</i>	This study
HMY345	W303-1a <i>rfa2-1::TRP1 HIS3::rfa2-214^b</i>	This study
HMY350	W303-1a <i>rfa2-1::TRP1 HIS3::rfa2-215^b</i>	This study
HMY346	W303-1a <i>rfa2-1::TRP1 HIS3::rfa2-C100^b</i>	This study
HMY353	W303-1a <i>rfa3-1::TRP1 HIS3::rfa3-313^b</i>	This study
HMY347	W303-1a <i>rfa3-1::TRP1 HIS3::rfa3-N70^b</i>	This study
RWY231	W303-1a <i>rfa2-1::TRP1 rfa3-1::TRP1</i> [pJM132 (<i>RFA1 RFA2 RFA3 URA3</i>)]	This study
RS192	W303-1a <i>top1-8 top2-1</i>	BRILL and STERNGLANZ (1987)

^a Extrachromosomal plasmids are indicated by brackets.

^b This strain has the indicated *rfa* allele integrated at *HIS3*.

or ammonium sulfate supplemented with lysine and other required amino acids, and 2 g/liter α -amino adipate (Sigma) as the sole nitrogen source (CHATTOO *et al.* 1979). Plates were incubated at room temperature for 6 days and the number of colonies counted. The mutation frequency was determined using the Method of the Median (LEA and COULSON 1948). The standard deviation of *m* was always $<0.4m$.

Microfluorometric analysis: Strains were grown in liquid YPD medium to early log phase and shifted to the nonpermissive temperature (37°) in a shaking water bath. Aliquots of cells were taken at 0, 2, 4, and 8 hr after shift, washed with 1 ml 50 mM sodium citrate, resuspended in 0.5 ml sodium citrate

containing 0.1 mg/ml RNase A and incubated at 37° for 2–4 hr. Propidium iodide was then added to a final concentration of 10 μ g/ml and incubated at 4° for up to 1 week. At least 20,000 cells per sample were analyzed on a Coulter-Epics fluorescence-activated cell sorter.

Immunoprecipitation and Western blot analysis: Yeast cultures were grown to early log phase and shifted to 37° at time zero. Ten-milliliter aliquots were taken at the indicated times and crude protein extracts prepared as follows. Cells were pelleted and resuspended in 0.2 ml RIPA buffer (20 mM Tris pH 8.0, 0.15 M NaCl, 0.5% deoxycholate, 1% Triton X-100 and 5 mM MgCl₂) and 0.1 ml dry glass beads were added.

TABLE 2

Plasmids used in this study

Name	Insert	Vector	Reference
pJM215	<i>RFA2^a</i>	pSK	BRILL and STILLMAN (1991)
pJM318	<i>RFA3</i>	pSK	BRILL and STILLMAN (1991)
pJM229	<i>RFA2</i>	pRS413 ^b	This study
pJM245	<i>RFA2</i>	pRS415	This study
pJM331	<i>RFA3</i>	pRS413	This study
pJM337	<i>RFA3</i>	pRS415	This study
pJM132	<i>RFA1/RFA2/RFA3</i>	pRS416	This study
pRT210	<i>rfa2-210</i>	pRS415	This study
pRT201-55	<i>rfa2-55</i>	pRS415	This study
pRT214	<i>rfa2-214</i>	pRS415	This study
pRT215	<i>rfa2-215</i>	pRS415	This study
pDP2C100	<i>rfa2-C100</i>	pRS415	PHILIPOVA <i>et al.</i> (1996)
pRT313	<i>rfa3-313</i>	pRS415	This study
pDP3N70	<i>rfa3-N70</i>	pRS415	PHILIPOVA <i>et al.</i> (1996)
YIp2-WT	<i>RFA2</i>	pRS403	This study
YIp2-210	<i>rfa2-210</i>	pRS403	This study
YIp2-55	<i>rfa2-55</i>	pRS403	This study
YIp2-214	<i>rfa2-214</i>	pRS403	This study
YIp2-215	<i>rfa2-215</i>	pRS403	This study
YIp2-C100	<i>rfa2-C100</i>	pRS403	This study
YIp3-313	<i>rfa3-313</i>	pRS403	This study
YIp3-N70	<i>rfa3-N70</i>	pRS403	This study
pHM313	<i>rfa3-313</i>	pRS413	This study
pHM3N70	<i>rfa3-N70</i>	pRS413	This study

^a All *RFA2* inserts are cDNA.

^b pRS vectors have been described (SIKORSKI and HIETER 1989).

Yeast cells were broken by vortexing (five times for 1 min, alternating with 1 min periods on ice) and the supernatant containing RPA was either immunoprecipitated by incubation with anti-RPA2 antibody (BRILL and STILLMAN 1991) (1:100 dilution) on ice for 1 hr followed by rocking with 50 μ l of protein-A sepharose beads (1:1 slurry in RIPA buffer) at 4° for 1 hr, or subjected to simple Western blot analysis as described (DIN *et al.* 1990). The immunoprecipitates were washed four times with RIPA buffer, resuspended in 20 μ l Laemmli buffer and resolved by 12% SDS-PAGE before transfer and immunoblotting with RPA1 antiserum.

DNA synthesis assay: DNA synthesis profiles were performed essentially as described (BRILL *et al.* 1987). Briefly, cells were grown in Y minimal medium with supplements as described (ZAKIAN *et al.* 1979) to a density of 7×10^6 cells/ml. The pH of the medium was then lowered to 4.5 with HCl and yeast α -factor (Sigma) was added to a final concentration of 20 μ g/ml. Cells were held in this medium for 1.5 doubling times to block the cells in G1 phase that was confirmed microscopically by the abundance of unbudded cells (>90%) and the appearance of schmoos. Cells were then pelleted, washed with Y medium and resuspended in fresh Y medium containing 20 μ Ci/ml [5,6]-³H uracil (ICN). One portion of the culture was grown at 25°, another at 37°, and part of the 25° culture was shifted to 37° after 90 min. The quality of the synchrony was verified microscopically: 78% of the arrested cells had small buds 90 min after release from the α -factor block. Aliquots (0.2 ml) were removed at the indicated times and placed in 0.5 ml stop solution (15% trichloroacetic acid, 200 μ g/ml thymine, and 50 mM sodium pyrophosphate) containing 0.1 ml of unlabeled stationary phase yeast cells as carrier. Pelleted cells were then resuspended in 50 μ l of 0.6 M NaOH and placed at 37° overnight for RNA hydrolysis. Samples were neutralized with 6 M HCl and precipitated with 1 ml stop solution on ice for 1 hr. Precipitates were collected on glass fiber filters and the filters were washed, dried, and the radioactivity determined by liquid scintillation counting.

RESULTS

Isolation of conditional-lethal *RFA2* and *RFA3* mutations: We had previously performed structure/function studies on RPA2 and RPA3 in yeast and found that both subunits contained large regions that were dispensible for viability and essential regions that resembled the ssDNA binding domains (SBD) of RPA1 (Figure 1A). To determine the *in vivo* function of these subunits a "plasmid shuffle" strategy and three methods of *in vitro* mutagenesis of cloned DNA was used to isolate seven conditional-lethal mutations in *RFA2* and *RFA3* (Figure 1B). First, truncated alleles of *RFA2* and *RFA3* that failed to grow at 37° were identified during the course of the structure/function studies mentioned above (PHILIPOVA *et al.* 1996). The *rfa2-C100* allele creates a stop codon at residue 174 resulting in a truncation of 100 C-terminal amino acids from RPA2. The *rfa3-N70* allele truncates 70 amino acids from the N-terminus of RPA3 by creating an initiating methionine at codon 70. In the second method we used site-directed mutagenesis of *RFA2* to change clusters of charged amino acids to alanine, a technique that has been shown to result in Ts⁻ phenotypes in yeast (WERTMAN *et al.* 1992). Of these 14 mutations, only the double point mutation E90A, D91A (*rfa2-55*) resulted in a Ts⁻

FIGURE 1.—Mutant *rfa2* and *rfa3* alleles. (A) Schematic illustration of the RPA2 and RPA3 proteins. Regions of the proteins that are dispensible for viability are represented by □. (B) List of *rfa2* and *rfa3* alleles used in this study. Also shown is the method by which they were created and the amino acid changes from wild-type.

phenotype. Finally, the *RFA2* and *RFA3* genes were subjected to PCR amplification under mutagenic conditions. Moderate levels of mutagenesis failed to result in Ts⁻ *rfa* mutants, perhaps because of the large nonessential regions of these proteins, so we increased the mutagenesis by extending the number of PCR cycles. As a result, we identified several Ts⁻ *rfa2* mutants, three of which were chosen for further study (Figure 1). We also identified a number of Ts⁻ *rfa3* mutants that displayed similar phenotypes and one (*rfa3-313*) was chosen for further characterization. As shown in Figure 1B, these PCR mutants contain multiple amino acid changes.

Each mutant gene was subcloned into pRS403 and integrated at the *HIS3* locus to create a stable single-copy transformant (Table 1). Compared to strains harboring mutant *rfa* alleles on plasmids, these strains showed noticeably lower levels of spontaneous reversion. These strains were judged to be sufficient for genetic analysis, as they did not give rise to colonies when streaked onto YPD medium and placed at the nonpermissive temperature (37°; Figure 2). At the permissive temperature (25°) all mutant strains grew noticeably slower than wild type (Table 3).

***rfa2* and *rfa3* mutants show defects in DNA repair and DNA replication fidelity:** To identify phenotypic differences between the *rfa* mutants, they were placed at the nonpermissive temperature for varying lengths of time, and then plated at the permissive temperature to determine the fraction of viable cells. These survival curves indicated a range of sensitivities, with many strains losing viability quickly at 37° (Figure 3A). The most severe killing was found in the *rfa2-214* and *rfa2-C100* strains, which showed only 1% viability after 6 hr at the nonpermissive temperature. Among the *rfa2* mutants, the *rfa2-55* and *rfa2-210* strains were the most

FIGURE 2.—*rfa2* and *rfa3* mutants fail to form colonies at 37°. Strains HMY343 (*rfa2-210*), HMY344 (*rfa2-55*), HMY345 (*rfa2-214*), HMY346 (*rfa2-C100*), HMY347 (*rfa3-N70*), HMY350 (*rfa2-215*), HMY353 (*rfa3-313*), and HMY357 (*RFA2 RFA3*) were streaked onto YPD plates and photographed after 3 days growth at the indicated temperature.

resistant, showing ~30% viability after 6 hr. Of the two *rfa3* mutants, *rfa3-N70* was very sensitive, losing over 90% viability in 6 hr, whereas *rfa3-313* showed no decrease in viability or increase in cell number at the nonpermissive temperature (Figure 3A).

Given the known requirement for human RPA in DNA repair (COVERLEY *et al.* 1991; HE *et al.* 1995; LI *et al.* 1995), we expected that the yeast *rfa* mutants might show DNA repair defects at the permissive temperature and tested them for sensitivity to DNA damaging agents. Most *rfa* strains are sensitive to UV when compared to wild type, but only at high doses of UV (Figure 3B). The *rfa2-210* mutant shows the greatest sensitivity to UV, but even in this case, it is more resistant than the well characterized *rad9* mutant. Nevertheless, we have recently demonstrated that the moderate level of UV sensitivity of the *rfa2-210* (aka *rfa2-210-3*) and *rfa2-215* (aka *rfa2-214-15*) mutants is sufficient to cause defects in nucleotide excision repair (NER) *in vitro* (HE *et al.*

1996). It is likely that the *rfa3* alleles, *rfa3-313* and *rfa3-N70*, are also defective in NER given their slight sensitivity to UV.

The *rfa* mutants exhibit a wide range of sensitivity to the alkylating agent MMS. As shown in Figure 3C, the *rfa2-215* mutant is the most sensitive strain, approaching the sensitivity of the *rad2* mutant. A second class of mutants (*rfa3-313*, *rfa2-210*, and *rfa2-55*) show an intermediate sensitivity, while the *rfa2-C100*, *rfa3-N70* and *rfa2-214* mutants are essentially resistant to MMS. We note that the sensitivity to DNA-damaging agents does not reflect the strains' thermosensitivity. For example, *rfa2-55* and *rfa3-313* are moderate to weak *Ts*⁻ mutants, but are very sensitive to MMS, and both highly thermosensitive truncation mutants appear to be resistant to MMS and only weakly sensitive to UV. Finally, the *rfa2-55* and *rfa2-210* mutants are sensitive to both UV and MMS. We conclude that these mutations display allele-specific differences in their response to DNA damaging agents and that these effects do not simply reflect the thermolability of the mutant RPA complex.

Because of the potential for RPA2 and RPA3 to control DNA replication fidelity, we tested the mutants for a mutator phenotype at the permissive temperature. We determined the frequency at which the strains could become resistant to α -aminoadipate, which most often selects for *lys2* mutations (CHATTOO *et al.* 1979). As shown in Table 3, many, but not all, *rfa* strains showed a mutator phenotype. Interestingly, the two most thermosensitive mutations, *rfa2-C100* and *rfa2-214*, showed a 10-fold difference in mutation frequency, with *rfa2-214* showing high-fidelity replication that was nearly as good as wild type. The weakly *Ts*⁻ *rfa3-313* displayed a rate fourfold greater than wild type. From the above assays we find that *rfa2-55* is the most consistently defective allele, showing a relatively high rate of mutation and high UV and MMS sensitivity.

TABLE 3

Mutator phenotype of *RFA2* and *RFA3* mutants

Strain	Doubling time ^a	Genotype	Mutation frequency ^b	Fold increase over wt
HMY357	133	W T	$7.3 \pm 3.0 \times 10^{-5}$	1
HMY347	171	<i>rfa3-N70</i>	$8.6 \pm 3.4 \times 10^{-5}$	1.2
HMY345	208	<i>rfa2-214</i>	$1.0 \pm 0.4 \times 10^{-4}$	1.4
HMY343	233	<i>rfa2-210</i>	$2.2 \pm 0.7 \times 10^{-4}$	3.0
HMY353	191	<i>rfa3-313</i>	$3.2 \pm 1.1 \times 10^{-4}$	4.4
HMY350	208	<i>rfa2-215</i>	$3.4 \pm 1.1 \times 10^{-4}$	4.7
HMY346	183	<i>rfa2-C100</i>	$8.0 \pm 2.4 \times 10^{-4}$	11
HMY350	224	<i>rfa2-55</i>	$9.6 \pm 2.9 \times 10^{-4}$	13

^a Doubling of optical density, in minutes, in YPD at 25°.

^b *LYS2* mutation rate (per cell per generation) and standard deviation, based on the frequency of α -aminoadipate-resistant colonies.

FIGURE 3.—Phenotypic analysis of *rfa2* and *rfa3* mutants. (A) Survival at 37°. Strains were grown to early log phase in YPD broth at 25°, incubated at 37° for the indicated times, and the percentage of viable cells determined by plating cells on YPD medium and counting the number of colonies after 3 days growth at 25°. (B) UV sensitivity. Strains were grown to early log phase in YPD broth, spread onto YPD plates, and immediately irradiated with the indicated dosage of UV light. Plates were incubated in the dark for 3 days at 25° and the percentage of viable cells determined by counting the number of colonies. (C) MMS sensitivity. Strains were grown to early log phase in YPD broth and incubated with 0.1% MMS for the indicated times before neutralization. The percentage of viable cells was determined as in A. Strains are HMY343 (*rfa2-210*), HMY344 (*rfa2-55*), HMY345 (*rfa2-214*), HMY346 (*rfa2-C100*), HMY347 (*rfa3-N70*), HMY350 (*rfa2-215*),

Cell-cycle arrest of *rfa2* and *rfa3* mutants at the non-permissive temperature: To further characterize phenotypic differences between the *rfa* mutants, we determined if cell growth arrested at a specific phase of the cell-cycle at the nonpermissive temperature. Exponentially growing strains were shifted to the nonpermissive temperature and aliquots were taken at 2-hr intervals and fixed for microfluorometric analysis. As shown in Figure 4, several *rfa2* mutants show preferential cell-cycle blocks. Most *rfa2* mutants (-210, -55, -215) accumulate primarily in the G2/M phase of the cell cycle as judged by this analysis. However, two mutants, *rfa2-214* and *rfa2-C100*, accumulate predominantly in G1. In the case of *rfa2-C100* there is a substantial G2/M population at 6 and 8 hr after the shift to nonpermissive temperature. This effect is unlikely to be due to mtDNA as very similar profiles were obtained when ρ^0 derivatives of these strains were analyzed (data not shown).

To confirm the above cell-cycle arrest points, we observed the cell morphology of the mutants after 8 hr at the nonpermissive temperature. As shown in Figure 5, strains judged to arrest primarily in G2 by microfluorometric analysis (*rfa2-210*, -55, -215) arrest with a significant proportion of budded cells, indicative of a late S-phase or G2/M block. The *rfa2* mutants that were judged to accumulate in G1 (-214, -C100) both arrest primarily as unbudded cells. We conclude that there are at least two classes of *rfa2* mutants that display different terminal phenotypes.

Both *rfa3* mutants arrest at the nonpermissive temperature primarily in the G1 phase of the cell cycle, but with significant G2/M populations, as judged by microfluorometric analysis (Figure 4). Like the *rfa2-C100* strain, both *rfa3* mutants display an initial increase in the G2/M population, followed by an increase in G1 population at late times after the temperature shift. Consistent with these results, *rfa3-N70* cells arrest predominantly as unbudded cells while most *rfa3-313* cells arrest with very small, or no buds (Figure 5).

Effect of the *rfa* mutations on the stability of the RPA complex: We suspected that the rapid Ts⁻ phenotype of the *rfa* mutants could be due to one of three events. In the first case, mutant RPA2 or RPA3 subunits might partially denature at the nonpermissive temperature, leaving the trimeric complex intact but disrupting essential interactions between the subunits, or between RPA and other factors. In the second case, mutant RPA2 or RPA3 subunits might denature and become separated from the RPA trimer at the nonpermissive temperature, leaving the other subunits nonfunctional. Finally, mutant RPA2 or RPA3 subunit might denature at the nonpermissive temperature, leading to degrada-

HMY353 (*rfa3-313*), and HMY357 (*RFA2 RFA3*). Each experiment was performed at least twice and each data point was measured in duplicate. A minimum of two dilutions were used in A and C. The figures present the averages obtained from all experiments.

DNA Content

FIGURE 4.—Microfluorometric analysis of *rfa2* and *rfa3* mutant strains. Each of the strains HMY343 (*rfa2*-210), HMY344 (*rfa2*-55), HMY345 (*rfa2*-214), HMY346 (*rfa2*-C100), HMY347 (*rfa3*-N70), HMY350 (*rfa2*-215), HMY353 (*rfa3*-313), and HMY357 (*RFA2 RFA3*) was grown to early log phase in YPD broth at 25° and shifted to 37°. Samples were taken at the indicated times, fixed, stained with propidium iodide and counted by FACS.

tion of the other subunits, including the known ssDNA binding subunit, RPA1. To distinguish among these possibilities we examined the effect of the shift to non-permissive temperature on the stability of the RPA complex using immunoprecipitation and Western blot (IP-Western) analysis.

FIGURE 5.—Terminal phenotype of *rfa2* and *rfa3* strains. Each of the strains HMY343 (*rfa2*-210), HMY344 (*rfa2*-55), HMY345 (*rfa2*-214), HMY346 (*rfa2*-C100), HMY347 (*rfa3*-N70), HMY350 (*rfa2*-215), HMY353 (*rfa3*-313), and HMY357 (*RFA2 RFA3*) was grown to early log phase in YPD broth at 25° and shifted to 37°. Samples were taken at 8 hr after shift, fixed and examined by phase contrast microscopy. Magnification, 600×.

Extracts were made from cultures grown at 25° and 37° and RPA was immunoprecipitated with RPA2 antiserum followed by Western blotting with RPA1 antiserum. Wild-type cells show a strong p69 IP-Western signal that increases after 2, 4, and 8 hr at 37° (Figure 6, top). This rise in signal is probably due to the increase in cell number during the course of the experiment. As expected, the p69 subunit in these wild-type extracts is stable at 37° as judged by a simple Western blot (Figure 6, bottom). In contrast, the following four mutants show reduced p69 IP-Western signal at the nonpermissive temperature: *rfa2*-55, -210, -214, and -C100. In three of these mutants (*rfa2*-55, -210, -214), the p69 subunit was judged to be stable by the parallel p69 Western blot (Figure 6). Further, probing the IP-Western blot with RPA2 antiserum indicated that the mutant p36 protein in these three strains was present and immunoprecipitated at the nonpermissive temperature, verifying that it had not degraded (data not shown). Therefore, in these three mutants the RPA1/RPA2 complex is weakened or dissociated at the nonpermissive temperature (case 2, above; simple complex dissociation). In the

FIGURE 6.—Stability of the RPA1/RPA2 complex. Each of the indicated strains was grown to early log phase in YPD broth at 25° and shifted to 37°. Samples were taken at the indicated times and a protein extract prepared. A portion of the extract was subjected to immunoprecipitation with RPA2 antiserum and the IP separated by SDS-PAGE and analyzed by Western blot with RPA1 antiserum (top). Another portion of the extract was simply subjected to Western blot with RPA1 antiserum to determine the stability of the RPA1 subunit at 37° (bottom). Strains are HMY343 (*rfa2-210*), HMY344 (*rfa2-55*), HMY345 (*rfa2-214*), HMY346 (*rfa2-C100*), HMY347 (*rfa3-N70*), HMY350 (*rfa2-215*), HMY353 (*rfa3-313*), and HMY357 (*RFA2 RFA3*).

rfa2-C100 mutant, however, the p69 polypeptide was susceptible to degradation at the nonpermissive temperature, indicating that the Ts^- defect in this strain may be due in part to loss of the p69 subunit (case 3, above; complex dissociation and subunit degradation). Finally, the *rfa2-215*, *rfa3-N70*, and *rfa3-313* mutants show a relatively strong p69 IP-Western signal that increases slightly during the course of the experiment. Thus, even though viability is lost at 37°, the RPA1/RPA2 complex remains intact in these three mutants (case 1, above; intact but inactive complex).

A fast-stop DNA synthesis phenotype in some *rfa2* mutants: To determine if the thermosensitivity of the *rfa* mutants was due to a direct effect on DNA replication, we measured ongoing DNA synthesis in the *rfa* mutants at the nonpermissive temperature. Cultures of each mutant were synchronized by α -factor arrest and released into synthetic medium containing 3H -uracil under the following conditions: one portion of the culture was released at 37°, a second portion was released at 25°, and finally, a portion of the 25° culture was shifted to 37° after 1.5 hr to determine the effect of inactivating RPA2 and RPA3 during S-phase. Aliquots of each culture were taken at intervals and processed to measure the incorporation of radioactive precursor into DNA.

DNA synthesis profiles indicate that each of the mutant cultures display normal kinetics of DNA synthesis at the permissive temperature (Figure 7). Initially, there is a lag phase during which the cells recover from α -factor arrest and enter G1. The synthetic phase then appears as a sharp increase in incorporated radioactivity followed by a plateau and then another round of DNA synthesis. During the first S-phase, 10,000–16,000 cpm of 3H -precursor are incorporated into DNA in ~3 hr. In contrast, when some of the mutant strains are released directly at 37°, only a small amount of DNA synthesis is observed (Figure 7). Using this protocol the *rfa2-210*, -55, -214, and -C100 mutant strains incorporate only 1000–4000 cpm over the course of the experiment, some of which is due to mtDNA replication. The lack of DNA synthesis could be due to defects in initiation, elongation, or both. To distinguish between these possibilities, a shift to 37° was performed within S-phase. Upon shift to 37°, these four *rfa2* mutants abruptly cease DNA synthesis. The DNA synthesis profile of a *top1 top2* double mutant strain is presented for comparison (Figure 7H), as this strain is known to be defective in replication fork movement at the nonpermissive temperature (BRILL *et al.* 1987; KIM and WANG 1989). We conclude that the DNA synthesis profiles of these four *rfa2* mutants are comparable to that of the *top1 top2* strain and that these strains arrest fork movement at the nonpermissive temperature.

A more subtle effect on DNA synthesis is observed with the *rfa2-215* allele. When this strain is released at 37°, it is able to undergo two rounds of DNA synthesis. Moreover, the shift from 25 to 37° does not seem to affect its rate of DNA synthesis; the first round is completed with normal kinetics and a second round yields twice that amount of synthesis. Thus, *rfa2-215* is not grossly defective in the initiation or elongation of DNA replication.

In the case of the *rfa3* mutants, cultures released at either 25° or 37° accumulate ~16,000 cpm of DNA before reaching a plateau. These mutants are therefore not defective in initiation. Nor does shifting the *rfa3* cultures to 37° within S-phase affect the kinetics of DNA synthesis. Unlike the *rfa2-215* mutation, however, the *rfa3* mutants do not undergo a second round of DNA synthesis at the nonpermissive temperature. The *rfa3* mutants complete only one round of synthesis, after which they divide and arrest primarily in G1 (see Figures 4 and 5). The *rfa3* mutants resemble the *rfa2-215* mutation in that they do not grossly affect initiation or fork movement, but are different in that they are unable to enter the next cell cycle.

Interactions between *rfa2* and *rfa3* alleles: To determine whether the *rfa2* and *rfa3* mutations showed allele-specific interactions, we tested whether they were synthetically lethal when present in the same cell at the permissive temperature. Strain RWY231 contains the *rfa2-1::TRP1 rfa3-1::TRP1* double disruption and is

FIGURE 7.—DNA synthesis profiles. Each of the indicated strains was grown to mid log phase in Y minimal medium at 25°, arrested with α -factor and released into medium containing [5,6]- ^3H -uracil under the following conditions. One sample was released and maintained at 37° (○—○) and a second sample was released and maintained at 25° (●—●). After 1.5 hr (arrow) a portion of the 25° sample was shifted to 37° (■—■). Aliquots were taken at the indicated times and the number of alkali-resistant TCA-precipitable counts determined. Strains are as follows: A, HMY343 (*rfa2-210*); B, HMY344 (*rfa2-55*); C, HMY345 (*rfa2-214*); D, HMY350 (*rfa2-215*); E, HMY346 (*rfa2-C100*); F, HMY353 (*rfa3-313*); G, HMY347 (*rfa3-N70*); H, RS192 (*top1-8 top2-1*).

complemented by a single *URA3* plasmid containing each of the wild-type *RFA* genes. We transformed RWY231 with all pairwise combinations of *rfa2* *LEU2* plasmids and *rfa3* *HIS3* plasmids (Table 2) and streaked

the transformants onto medium containing FOA. Almost all conditional alleles of *RFA2* were inviable in strains harboring the *rfa3-313* or *rfa3-N70* mutations, suggesting that *RFA2* and *RFA3* may be partially redun-

dant (data not shown). Only in a single case, the *rfa2-214 rfa3-313* transformant, was the double mutant viable. It is likely that these two alleles are functionally distinct and capable of producing mutant proteins that are together able to make essential interactions at the permissive temperature.

DISCUSSION

We report the isolation and characterization of conditional-lethal mutations in *RFA2* and *RFA3*, which encode the two small subunits of Replication Protein-A. Based on the cell-cycle-dependent phosphorylation of RPA2 and the absence of any biological activity, these subunits are thought to be regulatory proteins that might control the ssDNA binding activity of the largest subunit, RPA1 (DUTTA and STILLMAN 1992; FOTEDAR and ROBERTS 1992; CARDOSO *et al.* 1993). With the recent identification of potential ssDNA binding domains in these subunits, it is possible that they may play a direct role in binding ssDNA during DNA replication (PHILIPOVA *et al.* 1996). Our results describing the effects of mutations affecting these subunits strengthen this hypothesis.

Our data indicate that RPA2 is essential for the elongation phase of DNA replication. DNA synthesis studies in synchronized cultures revealed that most conditional alleles of *RFA2* cause an immediate cessation of DNA synthesis at the nonpermissive temperature (a "fast-stop" phenotype) reminiscent of the block to elongation seen in the absence of a topological swivel (BRILL *et al.* 1987; KIM and WANG 1989). Although our experiments do not directly address the initiation phase, no DNA synthesis was observed when these strains were released from G1 arrest directly at the nonpermissive temperature. This result is consistent with a requirement for RPA2 in initiation, but it might also be expected in the absence of any significant elongation. Further studies will be needed to confirm an *in vivo* role for RPA2 and RPA3 in the initiation of DNA replication. In contrast to these *rfa2* alleles, neither *rfa3* allele had a drastic effect on the initiation or elongation of DNA synthesis at the nonpermissive temperature. The *rfa3* mutants initiated and completed one round of DNA replication at the nonpermissive temperature before synthesis terminated (a "slow-stop" phenotype). At late times *rfa3* mutant cells were found primarily in the G1 phase of the cell cycle. While it is possible that the *rfa3* mutants are defective in the initiation of DNA replication in the subsequent cell cycle, we believe this is unlikely given their rapid loss of viability at 37° and their ability to initiate and complete the first round of synthesis at the nonpermissive temperature. Why then are these strains inviable? We suggest that these mutants accumulate incomplete replication products at the nonpermissive temperature and escape the G2 checkpoint to be recognized by a G1 checkpoint. Certain errors, such as double-stranded breaks, might occur during S

phase that are refractory to repair upon return to the permissive temperature.

Our data implicate both RPA2 and RPA3 in the process of DNA repair. Most *rfa2* and *rfa3* mutants displayed an increased sensitivity to DNA damaging agents, but allele-specific differences in the degree of sensitivity and a selective response to the two agents suggest that specific repair pathways may be affected in the various mutants. For example, the *rfa3-313* and *rfa2-215* alleles are only moderately UV-sensitive but display the greatest sensitivity to MMS, while two other moderately UV-sensitive alleles, *rfa2-C100* and *rfa3-N70*, showed no increased sensitivity to MMS. This repair defect has been confirmed biochemically for some alleles. Nucleotide excision repair *in vitro* is reduced in extracts made from *rfa2-210* and *rfa2-215* strains and this defect can be complemented by the addition of exogenous wild-type RPA (HE *et al.* 1996). The *rfa2* and *rfa3* alleles that show specific repair defects should permit a genetic analysis to identify interacting components in these repair pathways.

A number of phenotypes that we examined in the *rfa2* and *rfa3* mutants displayed allele-specific differences. First, *rfa2-215* was unique among the *rfa2* mutants in that it did not display a fast-stop replication phenotype and was able to complete two rounds of replication at the nonpermissive temperature. We think it is unlikely that *rfa2-215* is leaky given its rapid loss of viability at 37°. We suspect that the lethal defect in this mutant is similar to that of the *rfa3* mutants: poor fidelity of replication is exacerbated at 37°, leading to excessive DNA damage that, in this case, arrests in G2. Second, while DNA replication mutants are expected to arrest at this phase of the cell cycle, not all mutants showed this terminal phenotype. The *rfa2-214* and *rfa2-C100* are fast-stop mutants, but arrest with significant G1 populations, like the *rfa3* mutants. How these mutants are able to escape the G2 checkpoint is unclear, but one possibility is that RPA2 and/or RPA3 is part of a G2 signalling pathway that is defective in these mutants. Finally, the *rfa2-214* allele is unique in that it displays a fast-stop phenotype at the nonpermissive temperature, but essentially wild-type fidelity at the permissive temperature. In this mutant, the roles of RPA2 in promoting replication fork movement and replication fidelity appear to be separated.

While this work was in progress, a report describing two *rfa2*Ts⁻ alleles appeared (SANTOCANALE *et al.* 1995). By coincidence, the amino acid changes in these two alleles (D91G and C173-STOP) closely resemble the changes in our *rfa2-55* and *rfa2-C100* alleles, respectively. We find essentially identical phenotypes in our mutants, except that the *rfa2-C100* allele accumulates primarily in G1 at the nonpermissive temperature, as opposed to the *rfa2-C173-STOP* allele, which blocks at G2/M. This inconsistency may be due to experimental differences such as the time of incubation at the nonpermissive temperature, or to strain background differ-

ences. The additional alleles that we have identified allowed us to test how RPA2 and RPA3 affect the stability of the RPA complex and the elongation phase of DNA replication.

We were struck by the correlation between the instability of the RPA1/RPA2 complex in fast-stop mutants and its stability in slow-stop mutants. We have shown that while the RPA1 subunit is present in three fast-stop mutants (*rfa2-55*, *rfa2-210*, *-C214*) at the nonpermissive temperature, it is poorly precipitated along with RPA2 subunit (Figure 6). We suggest that this result is due to a weakened or unstable RPA1/RPA2 complex at the nonpermissive temperature, since we know that p36 is present and recognized by the antiserum under these conditions. In the case of *rfa2-C100*, the p69 subunit degrades at the nonpermissive temperature, clearly resulting in loss of RPA activity. Interestingly, the fast-stop DNA synthesis phenotype of this mutant is no more severe than any other fast-stop mutant in which RPA1 is not degraded (Figure 7). Finally, three *Ts⁻* strains (*rfa2-215*, *rfa3-N70*, and *rfa3-313*) have a stable RPA1/RPA2 complex at the nonpermissive temperature and no defect in the elongation phase of replication.

While it is possible that RPA becomes unstable in the presence of a replication fork block, we believe that the simpler interpretation of our results is that some *Ts⁻* mutations cause RPA1 and RPA2 to dissociate, resulting in a block to replication fork movement. Interestingly, both of these subunits are proposed to bind ssDNA (PHILIPOVA *et al.* 1996). While the role of the multiple ssDNA binding subunits in RPA is unknown, we suggest that the interaction of ssDNA with both subunits is essential for replication fork movement. One possibility is that the multiple ssDNA binding domains wrap ssDNA about the RPA trimer in a higher-order mode during replication elongation. Such an effect is observed with ssDNA and *E. coli* SSB *in vitro* (LOHMAN and FERRARI 1994; RIGLER and ROMANO 1995). Alternatively, it is possible that the multiple ssDNA binding domains are employed during the displacement of the SSB (as the replication machinery passes) and the reloading of SSB (as the newly unwound DNA appears at the head of the replication fork). In this case, the multiple ssDNA binding domains may be used to tether the SSB molecules to different sites on the DNA so as to increase its effective local concentration. Either model is consistent with the requirement for multiple ssDNA binding domains in all known cellular SSBs (bacterial SSB, mtSSB, and RPA). Further studies of the RPA mutants in yeast will be of immense value in distinguishing between these alternative models.

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