

ATP Utilization by Yeast Replication Factor C

IV. RFC ATP-BINDING MUTANTS SHOW DEFECTS IN DNA REPLICATION, DNA REPAIR, AND CHECKPOINT REGULATION*[§]

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Replication factor C is required to load proliferating cell nuclear antigen onto primer-template junctions, using the energy of ATP hydrolysis. Four of the five RFC genes have consensus ATP-binding motifs. To determine the relative importance of these sites for proper DNA metabolism in the cell, the conserved lysine in the Walker A motif of RFC1, RFC2, RFC3, or RFC4 was mutated to either arginine or glutamic acid. Arginine mutations in all RFC genes tested permitted cell growth, although poor growth was observed for *rfc2-K71R*. A glutamic acid substitution resulted in lethality in RFC2 and RFC3 but not in RFC1 or RFC4. Most double mutants combining mutations in two RFC genes were inviable. Except for the *rfc1-K359R* and *rfc4-K55E* mutants, which were phenotypically similar to wild type in every assay, the mutants were sensitive to DNA-damaging agents. The *rfc2-K71R* and *rfc4-K55R* mutants show checkpoint defects, most likely in the intra-S phase checkpoint. Regulation of the damage-inducible *RNR3* promoter was impaired in these mutants, and phosphorylation of Rad53p in response to DNA damage was specifically defective when cells were in S phase. No dramatic defects in telomere length regulation were detected in the mutants. These data demonstrate that the ATP binding function of RFC2 is important for both DNA replication and checkpoint function and, for the first time, that RFC4 also plays a role in checkpoint regulation.

Replication factor C (RFC)¹ is the eukaryotic clamp loader that uses the energy of ATP hydrolysis to load the replication clamp proliferating cell nuclear antigen (PCNA) onto the DNA at a primer-template junction. RFC in yeast is a heteropentamer that consists of a large subunit (Rfc1, 95 kDa) and four small subunits (Rfc2–Rfc5, 36–40 kDa). The yeast Rfc subunits are all quite similar to each other at the amino acid level (24–37%) and to the human Rfc subunits. However, each yeast

subunit shares the highest degree of sequence homology with the analogous subunit from human RFC (reviewed in Ref. 1). The sequence similarity is localized to the N-terminal half of the subunits in regions termed RFC boxes II–VIII. Most notable are the putative ATP-binding motifs in boxes III and V. The amino acids in box III, the Walker A motif, create a characteristic fold, the P loop, which forms a pocket for binding of the β - and γ -phosphates of ATP (2, 3).

Our biochemical studies reported in the second paper (4) of this series show that four ATP molecules can bind to RFC when PCNA and primer-template DNA are also present. This coincides with our current understanding of the primary sequence determinants of ATP-binding domains, which indicates that four of the five subunits comprising the RFC complex contain consensus ATP-binding motifs. (see Fig. 1A). The Rfc5 subunit lacks critical residues in both the A and B motifs and therefore this domain may have a more structural function analogous to the δ' subunit of *Escherichia coli* DNA polymerase III holoenzyme (5).

Mutation of the conserved lysine residue to glutamic acid in the RFC1, RFC2, RFC3, or RFC4 gene resulted in mutant RFC complexes with varying defects in *in vitro* assays. Complete loss of clamp loading activity was observed in the RFC-2E complex (containing Rfc2-K71E), and the RFC-3E complex (containing Rfc3-K59E) displayed only marginal activity (6). The RFC-4E complex (containing Rfc4-K55E) showed primarily a severe K_m defect for ATP binding, and the biochemical studies indicated that ATP binding to the Rfc4 subunit is essential for clamp loading. Surprisingly, however, the RFC-1E complex (containing Rfc1-K359E) was essentially like wild type, contrasting sharply with reports of similar mutations in human RFC, which abrogated the clamp loading activity of the mutant complex, and suggesting that ATP binding to Rfc1 is not essential for clamp loading in yeast (6–8).

In addition to the role of RFC in DNA replication, several of its subunits appear to be involved in DNA repair (Ref. 9 and this study) as well as checkpoint regulation. The cells progress through the cell cycle in an orderly fashion with late events dependent upon the successful completion of early events. When incompletely replicated DNA or DNA damage is detected, a checkpoint is activated until DNA synthesis is complete or the damage has been repaired. Mutations in two components of the budding yeast RFC complex, RFC2 (10) and RFC5 (11, 12) and mutations in *Schizosaccharomyces pombe* *rfc2*⁺ (13) and *rfc3*⁺ (14) have revealed a role for RFC in checkpoint regulation. In addition, mutations in other DNA replication genes, namely the catalytic subunit of DNA polymerase ϵ , POL2 (15) and a polymerase ϵ associated gene DPB11 (16, 17), show defects in the S phase checkpoint.

Our biochemical analysis has shown that ATP binding to the

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains figures for the Appendix.

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¹ The abbreviations used are: RFC, replication factor C; Rfc2–5, complex of Rfc2p, Rfc3p, Rfc4p, and Rfc5p; Rfcx, xth subunit of RFC; PCNA, proliferating cell nuclear antigen; MMS, methylmethane sulfonate; bp, base pair.

TABLE I
Strains

For simplicity only viable strains are listed. All strains were created for this study except PY73, which was obtained from Hannah Klein.

PY73 (W303)	MAT α <i>ade2-1 his3-11,15 ura3-1 trp1-1 leu2-3,112 can1-100</i>
PY90	MAT α , as PY73, but Δ RFC2::kanMX6 + [pBL615]
PY90-K	PY90 + [pBL616] - [pBL615]
PY90-R	PY90 + [pBL621] - [pBL615]
PY91	MAT α , as PY73, but Δ RFC2::hisG + [pBL615]
PY91-K	PY91 + [pBL616] - [pBL615]
PY91-R	PY91 + [pBL621] - [pBL615]
PY92	MAT α , as PY73, but Δ RFC3::kanMX6 + [pBL617]
PY92-K	PY92 + [pBL618] - [pBL617]
PY92-R	PY92 + [pBL623] - [pBL617]
PY94	MAT α , as PY73, but Δ RFC4::kanMX6 + [pBL619]
PY94-K	PY94 + [pBL620] - [pBL619]
PY94-E	PY94 + [pBL626] - [pBL619]
PY94-R	PY94 + [pBL625] - [pBL619]
PY96-71R/55E	MAT α , as PY73, but Δ RFC2::hisG, Δ RFC4::kanMX6 + [pBL630-R] + [pBL626]
PY98-359R/71R	MAT α <i>ade2-1 his3-11,15 ura3 trp1 leu2</i> Δ RFC1::LEU2 Δ RFC2::kanMX6 + [pBL643-R] + [pBL621]
PY99-359R/59R	MAT α <i>ade2-1 his3-11,15 ura3 trp1 leu2</i> Δ RFC1::LEU2 Δ RFC3::kanMX6 + [pBL642-R] + [pBL632]
PY100-359R/55E	MAT α <i>ade2-1 his3-11,15 ura3 trp1 leu2</i> Δ RFC1::LEU2 Δ RFC4::kanMX6 + [pBL642-R] + [pBL633-E]
PY103	MAT α , <i>leu2Δ1 trp1Δ63 ura3-52</i> Δ RFC1::LEU2 + [pBL641]
PY103-K	PY103 + [pBL642] - [pBL641]
PY103-E	PY103 + [pBL642-E] - [pBL641]
PY103-R	PY103 + [pBL642-R] - [pBL641]

Rfc2, Rfc3, and Rfc4 subunits is required for clamp loading, whereas ATP binding to the Rfc1 subunit is dispensable for this process *in vitro*. To determine whether these properties reflect those inside the cell or whether ATP binding to Rfc subunits drives alternative pathways, we have carried out a complementary genetic analysis of RFC ATP-binding mutants. We have examined the *in vivo* phenotypes of both Lys \rightarrow Glu and Lys \rightarrow Arg mutations in the ATP-binding motifs of the *RFC1*, *RFC2*, *RFC3*, and *RFC4* genes. The results of the genetic studies indicate that intact ATP-binding motifs are critical in *RFC2* and *RFC3*. *RFC2* and *RFC4* mutants are defective in response to DNA damage, indicating roles for these subunits in checkpoint regulation.

EXPERIMENTAL PROCEDURES

Strains—The yeast strains used in this study are listed in Table I. Standard growth media and yeast genetic techniques were used. *RFC2*, *RFC3*, and *RFC4* deletion strains were created in the diploid strain W303 (a gift from H. Klein). This strain was renamed PY73. PY90 and PY91 were created by transforming PY73 with a *SalI/NheI* digest of plasmids pBL628 and pBL627A, respectively. Complementing plasmid pBL615 was transformed into the diploid transformants, and appropriate spores were collected. PY92 was made by transforming PY73 with a *BsiWI/PvuII* digest of plasmid pBL629A, followed by transformation with pBL617 and spore collection. PY94 was created in PY73 using a polymerase chain reaction disruption method with pFA6a-kanMX6 (a generous gift from A. Wach) (18). pBL619 was transformed into the diploid strain, and appropriate spores were collected. PY96-K71R/K55E was created by mating PY91 with PY94-E, sporulating, and identifying the appropriate haploids, followed by transformation with pBL630-R and a plasmid shuffle on 5-fluoroorotic acid. PY103 is strain CH1578 (a generous gift from C. Holm) that contains pBL641 in place of pCH1160. PY98-K359R/K71R was created by mating PY103 with PY90-R, sporulating, and identifying the appropriate his⁻ haploids, followed by transformation with pBL643-R and a plasmid shuffle on 5-fluoroorotic acid. PY99-K359R/K59R was created by mating PY103 with PY92-R, sporulating, and identifying the appropriate his⁻ haploids. The strain was transformed with pBL632 followed by growth to identify cells that had lost the *TRP1* plasmid (pBL623). Finally, the strain was transformed with pBL642-R and a plasmid shuffle on 5-fluoroorotic acid was performed. PY100-359R/K55E was created by mating PY103 with PY94-E, sporulating, and identifying the appropriate his⁻ haploids. The strain was transformed with pBL633-E followed by growth to identify cells that had lost the *TRP1* plasmid (pBL626). Finally, the strain was transformed with pBL642-R, and a plasmid shuffle on 5-fluoroorotic acid was performed. All strains were confirmed by Southern analysis. *E. coli* strains used include DH5, ABLE C (Stratagene), XL1-Blue, and BW313 (dut ung).

Plasmids—The plasmids used throughout this study are listed in

TABLE II
Plasmids

Only the relevant genotypes are given. For details see "Experimental Procedures."

pBL412	2 μ M-ori, <i>GAL1/10-RFC2, URA3</i>
pBL412-R	2 μ M-ori, <i>GAL1/10-rfc2-K71R, URA3</i>
pBL412-E	2 μ M-ori, <i>GAL1/10-rfc2-K71E, URA3</i>
pBL413	2 μ M-ori, <i>GAL1/10-RFC3, LEU2</i>
pBL413-R	2 μ M-ori, <i>GAL1/10-rfc3-K59R, LEU2</i>
pBL413-E	2 μ M-ori, <i>GAL1/10-rfc3-K59E, LEU2</i>
pBL615	<i>RFC2, URA3, CEN6, ARSH4</i>
pBL617	<i>RFC3, URA3, CEN6, ARSH4</i>
pBL621	<i>rfc2-71R, TRP1, CEN6, ARSH4</i>
pBL623	<i>rfc3-59R, TRP1, CEN6, ARSH4</i>
pBL625	<i>rfc4-55R, TRP1, CEN6, ARSH4</i>
pBL626	<i>rfc4-55E, TRP1, CEN6, ARSH4</i>
pBL630-R	<i>rfc2-71R, LEU2, CEN6, ARSH4</i>
pBL632	<i>rfc3-59R, HIS3, CEN6, ARSH4</i>
pBL633-E	<i>rfc4-55E, HIS3, CEN6, ARSH4</i>
pBL641	<i>RFC1, URA3, CEN6, ARSH4</i>
pBL642-R	<i>rfc1-359R, TRP1, CEN6, ARSH4</i>
pBL642-E	<i>rfc1-359E, TRP1, CEN6, ARSH4</i>
pBL643-R	<i>rfc1-359R, HIS3, CEN6, ARSH4</i>
pBL210	<i>POL30 TRP1</i> 2 μ M-ori

Table II. pBL641 (*RFC1, URA3*) is a complementing plasmid containing the entire *RFC1* sequence and was created by cloning a 922-bp *BamHI/BglII* fragment containing the N terminus of *RFC1* into the *BamHI* site of plasmid pCH1160 (19). pBL642 (*RFC1, TRP1*) was created by cloning the 5364-bp *PvuI* fragment from pBL641 into the 2871-bp *PvuI* fragment of pRS314. pBL642-R and pBL642-E (*rfc1-K359R* or *rfc1-K359E, TRP1*) were created by annealing mutagenic primers (1K359R, 5'-CCTGGTATTGGGCGTACGACTGCTGCTC-3'; 1K359E, 5'-CCTGGTATTGGGCGAGACAACGACTGCTGCTC-3'; mutations are underlined) to single-stranded, circular, uracil-containing pBL642 DNA (20) and extending the primers with T7 DNA polymerase, followed by ligation. These extension reactions were transformed into *E. coli* strain XL1-Blue. Isolates carrying the mutations were identified by digestion of plasmid DNA with *BsiWI* (K359R) or *BsmAI* (K359E). Isolates identified by restriction analysis were confirmed by sequencing. pBL643-R (*rfc1-K359R, HIS3*) was created by cloning the 5365-bp *PvuI* fragment from pBL642 into the 3054-bp fragment of pRS313. pBL643-E (*rfc1-K359E, HIS3*) was created in yeast as follows. pBL642-E was cotransformed into yeast strain PY73 with the 4522-bp *PvuII* fragment from pRS313. DNA was purified from the his⁺ transformants and was transformed into *E. coli* strain ABLE C followed by confirmation of the plasmid by restriction analysis.

pBL615 (*RFC2, URA3*) and pBL616 (*RFC2, TRP1*) are complementing plasmids created by cloning the 2557-bp *EcoRI/SalI* fragment of pBS-RFC2 (a gift from A. Sugino) into the *EcoRI/SalI* sites of pRS316 and pRS314, respectively. pBL621 (*rfc2-K71R, TRP1*) and pBL622

(*rfc2-K71E*, *TRP1*) were created by annealing mutagenic primers (2K71R, 5'-CCAGGAACCTGGTCGTACGTCTACC-3'; 2K71E, 5'-CCAGGAACCTGGTGAACCTGTACC-3'; mutations are underlined) to single-stranded, circular, uracil-containing pBL616 DNA, followed by the treatment described above. Correct isolates were detected by sequencing (K71R) or by digestion with *HphI* (K71E) followed by sequencing. pBL630-R (*rfc2-K71R*, *LEU2*) was created by cloning the 2563-bp *SalII/PstI* fragment from pBL621 into the *SalII/PstI* sites of pRS315.

pBL617 (*RFC3*, *URA3*) and pBL618 (*RFC3*, *TRP1*) are complementing plasmids that were created by cloning the 3085-bp *EcoRI/BglII* fragment of pBL452 (24) into the *EcoRI/BamHI* sites of pRS316 or pRS314, respectively. pBL623 (*rfc3-K59R*, *TRP1*) and pBL624 (*rfc3-K59E*, *TRP1*) were created by annealing mutagenic primers (3K59R, 5'-CAATTGTAGAAGTACGACCGGTACCTGG-3'; 3K59E, 5'-CAATTGTAGAAGTTTCCCCTGGTACCTGG-3'; mutations are underlined) to single-stranded, circular, uracil-containing pBL618 DNA, followed by the treatment described above. Correct isolates were identified by digestion with *BsiEI* (K59R) or *BsaJI* (K59E) followed by sequencing. pBL632 (*Rfc3-K59R*, *HIS3*) was created by cloning the 4981-bp *PvuII* fragment from pBL623 into the 3054-bp *PvuII* fragment of pRS313.

pBL619 (*RFC4*, *URA3*) and pBL620 (*RFC4*, *TRP1*) are complementing plasmids that were created by cloning the 2241-bp *BstEII* (filled)/*XhoI* fragment from pBL551 (a 7.5-kb *BamHI* yeast genomic fragment containing the *RFC4* locus cloned into pUC19) into the *EcoRI* (filled)/*XhoI* sites of pRS316 and pRS314, respectively. pBL625 (*rfc4-K55R*, *TRP1*) and pBL626 (*rfc4-K55E*, *TRP1*) were created by annealing mutagenic primers (4K55R, 5'-CCAGGTATAGGTCGTACCACTTCGGTAC-3'; 4K55E, 5'-CCAGGTATAGGTGAGACCACTTCGGTAC-3'; mutations are underlined) to single-stranded, circular, uracil-containing pBL620 DNA, followed by the treatment described above. Correct isolates were identified by sequencing. pBL633-R (*rfc4-K55R*, *HIS3*) and pBL633-E (*rfc4-K55E*, *HIS3*) were created by cloning the 4126-bp *PvuI* fragment from pBL625 or pBL626, respectively, into the 3054-bp *PvuI* fragment of pRS313.

pBL412, pBL413, pBL424, and pBL425 have been described previously (16). Plasmids were created such that the *RFC2* and *RFC3* mutants could be overproduced under control of the *GAL1/10* promoter. pBL412-R (*GAL1/10-rfc2-K71R*, *URA3*) and pBL412-E (*GAL1/10-rfc2-K71E*, *URA3*) were created by replacing the 338-bp *PfIMI/NheI* in pBL412 with the same fragment from pBL621 or pBL622, respectively. pBL413-R (*GAL1/10-rfc3-K59R*, *LEU2*) and pBL413-E (*GAL1/10-rfc3-K59E*, *LEU2*) were created by replacing the 397-bp *MscI/NcoI* fragment of pBL413 with the same fragment from pBL623 or pBL624, respectively.

pBL627A contains the *RFC2* gene, which has been disrupted by inserting a hisG-*URA3*-hisG cassette into the *NdeI/HpaI* sites of *RFC2* in pBL616. pBL628 contains the *RFC2* gene, which has been disrupted by inserting the *kanMX6* gene into the *NdeI/HpaI* sites of *RFC2* in pBS-RFC2.

Plasmid pBL457 contains the *RFC3* gene, which has been disrupted by inserting a hisG-*URA3*-hisG cassette into the *EcoNI/NcoI* sites of *RFC3* in plasmid pBL452 (2). pBL629 contains the *RFC3* gene, which has been disrupted by inserting the *kanMX6* gene (50) into the *EcoNI/NcoI* sites of *RFC3* in pBL452.

DNA Damage Sensitivity Measurements—Sensitivity of the mutants to growth on methyl methanesulfonate (MMS) or MMS survival curves, sensitivity of the mutants to growth on hydroxyurea-containing medium, or sensitivity to UV irradiation was determined as described previously (21).

RNR3 Induction—Plasmid pZZ2 (*RNR3-lacZ*, *URA3*, *CEN4*, *ARS1*) was a gift from S. Elledge. *RNR3* induction assays were carried out essentially as described (22). *RFC* deletion strains, complemented by wild type or mutant *RFC* genes on *TRP1* plasmids plus plasmid pZZ2, were treated with 0.01% MMS for 5 h at 30 °C. All assays were done in triplicate and were repeated three to five times.

Protein Extracts—The mutant strains and isogenic wild type controls were grown in YPD medium at 30 °C to OD₆₆₀ = 2. In some experiments 100 mM hydroxyurea was added to the cultures at an OD₆₆₀ = 0.5, and growth continued at 30 °C until OD₆₆₀ = 2 (6–9 h). Protein extracts were made from 4-ml cultures essentially as described and were generally at 1 mg/ml (23). Extracts were fractionated on 10% acrylamide gels, transferred to nitrocellulose, and blotted with immunopurified Rfc antibodies as detailed in the legends to the figures. Detection was with either with an ECL kit from Amersham Pharmacia Biotech or with an alkaline phosphatase-conjugated secondary antibody followed by staining with the BCIP/NBT FAST substrate from Sigma.

To measure phosphorylation of Rad53p, early log phase cells were arrested with 10 μg/ml α-factor (Sigma) for 2 h. Twenty minutes after

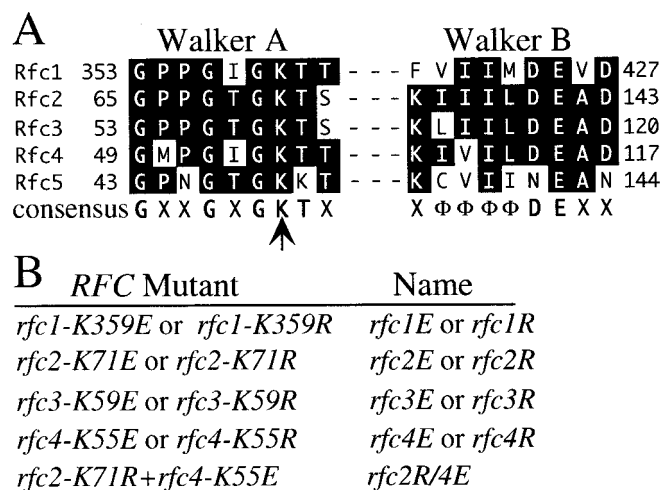


FIG. 1. Comparison of ATP-binding domains in RFC genes. A, the Walker A and B motifs are shown. The consensus indicates critical residues within these domains as defined by Neuwald *et al.* (47). X is any amino acid, and Φ is a hydrophobic residue. In this study, the conserved lysine in the Walker A motif (arrow) was altered to arginine or glutamic acid. B, for simplicity, the strain names will be abbreviated in figures and tables as shown.

release from α-factor, MMS was added to 0.1% final concentration, and time points were taken. Extracts were fractionated on 7.5% acrylamide gels, transferred to nitrocellulose, and blotted with an anti-Rad53 antibody (yC-19) from Santa Cruz Biotechnology. Detection was with an ECL kit from Amersham Pharmacia Biotech.

RESULTS

Viability of ATP-binding Domain Mutants—Site-directed mutations were created in the ATP-binding motifs of the *RFC1*, *RFC2*, *RFC3*, and *RFC4* genes. The Rfc5 subunit lacks critical residues in both the Walker A and B motifs of the ATP-binding domain and was therefore not subjected to mutagenesis (Fig. 1A). As with the δ' subunit of the *E. coli* γ-complex, the ATP-binding domain in Rfc5p likely is of structural importance rather than of importance in binding ATP (5). The conserved lysine in the Walker A motif was mutated to either an arginine or glutamic acid residue (Fig. 1A). Arginine was chosen because in some instances a Lys → Arg mutation still permits ATP binding, although in general the protein is biologically inactive (24–26). The reversal of charge in the Lys → Glu mutants was expected to virtually abolish ATP binding and ATPase activity. Our biochemical analysis also showed a more severe phenotype for the Lys → Glu mutations than for the analogous Lys → Arg mutations (6). Fig. 1B outlines the abbreviations used for the mutant strains throughout this paper.

A chromosomal deletion of each particular *RFC* was created as described under "Experimental Procedures." Because all *RFC* genes are essential, the chromosomal deletion was complemented by the wild type *RFC* gene on a centromere plasmid containing also the *URA3* gene as a selectable marker. These plasmids, as well as the ones described below, contained the wild type or mutant *RFC* gene positioned behind its natural promoter to ensure appropriate expression. A test of the different wild type strains showed that they were similar to the parental strain (W303) in growth and response to DNA-damaging agents (data not shown). The appropriate strain with the wild type *RFC* gene on a *URA3* plasmid was transformed with a second centromere plasmid with either *TRP1*, *HIS3*, or *LEU2* as a selectable marker and carrying either the wild type *RFC* gene or the Lys → Arg or Lys → Glu mutant gene, and cells containing both plasmids were plated on 5-fluoroorotic acid-containing medium at 13, 23, or 30 °C. The cells that were able to grow on this medium had lost the *URA3* plasmid containing

TABLE III
Growth and repair defects of the mutants

For growth at 13 or 30 °C, 10⁵, 10⁴, 10³, and 10² cells were spotted as 10- μ l drops on YPD plates and growth was scored after 6–8 or 2–3 days, respectively. Growth on YPD plates containing 110 mM hydroxyurea or 0.02% methyl methanesulfonate was determined after 3–4 days at 30 °C. Sensitivity to ultraviolet light was determined after plating of the cells and irradiation of the plates at 100 J/m². Wild type survival was 44%. +++ indicates growth or survival like wild type; ++ \pm indicates minimal defects in growth or survival; ++, +, and +/- indicate that growth or survival similar to wild type was observed at approximately 10-, 100-, and 1000-fold greater cell amounts, respectively; - indicates that no growth was observed.

Mutant	13 °C	30 °C	Hydroxyurea	MMS	UV
Wild type	+++	+++	+++	+++	+++
<i>rfc1R</i>	+++	+++	+++	+++	+++
<i>rfc1E</i>	++ \pm	++ \pm	++ \pm	++ \pm	++ \pm
<i>rfc2R</i>	+	+	-	-	++
<i>rfc2E</i>	lethal				
<i>rfc3R</i>	+++	+++	+++/-	++ \pm	++ \pm
<i>rfc3E</i>	lethal				
<i>rfc4R</i>	+	+++	-	++	++
<i>rfc4E</i>	+++	+++	+++	+++	+++
<i>rfc1R/2R</i>	+/-	+	-	-	++
<i>rfc1R/3R</i>	+++	+++	+	++	+++
<i>rfc1R/4R</i>	lethal				
<i>rfc1R/4E</i>	+/-	++	+	+++	+
<i>rfc1E/2R</i>	lethal				
<i>rfc1E/3R</i>	lethal				
<i>rfc1E/4R</i>	lethal				
<i>rfc1E/4E</i>	lethal				
<i>rfc2R/3R</i>	lethal				
<i>rfc2R/4R</i>	lethal				
<i>rfc2R/4E</i>	-	+	-	-	+
<i>rfc3R/4E</i>	lethal				
<i>rfc3R/4R</i>	lethal				

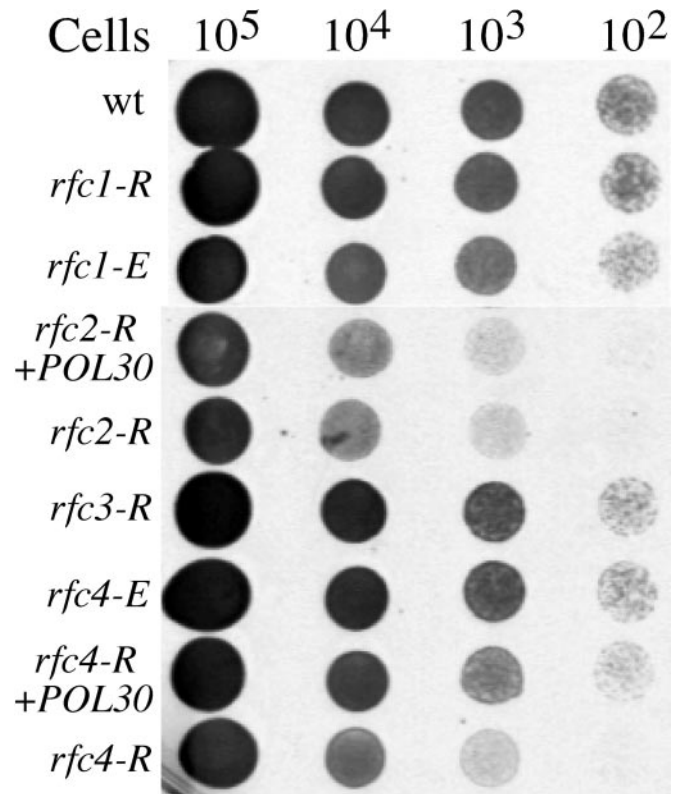


FIG. 2. Defects in mutant cell growth at 13 °C. See Table III and “Experimental Procedures” for details. +POL30 indicates that PCNA was overproduced about 20-fold in the strain. *wt*, wild type.

the wild type gene and were therefore able to grow with the RFC mutant gene. Colonies were replated on YPD plates, again at various temperatures, and growth was determined. Table III shows the results of these experiments, and Fig. 2 shows growth defects in the mutants at 13 °C.

For each RFC gene, when the lysine was replaced by an arginine, cell growth occurred. In fact, except for the *rfc2R* mutant, no growth defects were observed in the mutants at 30 °C. The *rfc2R* mutant strain grew very poorly on synthetic selection medium, but adequate growth was observed when the mutant was propagated on rich medium. In rich medium the *rfc2R* mutant had a plating efficiency of ~30% and a doubling time 1.6 times greater than wild type at 30 °C. An analysis by fluorescence microscopy of the mutant showed an accumulation of large budded cells. In ~60% of these arrested cells the nucleus was positioned at the neck of the bud, consistent with an arrest in the G₂/M phase of the cell cycle because of replication defects (data not shown). However, 40% of the cells did complete nuclear division. The low plating efficiency of the mutant and the incomplete cell cycle arrest are suggestive of a checkpoint defect in the *rfc2R* strain, in addition to the replication defect (see below). The *rfc4R* strain showed some cold sensitivity for growth.

When glutamic acid was substituted for lysine in RFC2 and RFC3 lethality resulted. In contrast, the *rfc1E* mutant had only a slight growth defect, and the *rfc4E* mutant had no detectable defects (Fig. 2 and Table III).

Expression of Mutant Rfc Subunits—An observed phenotypic defect in a particular RFC mutant may not necessarily be due to the inability of the mutant subunit to bind and/or hydrolyze ATP. For instance, defects could also result if the mutant subunit were unstable, not properly folded, failed to assemble into an appropriate RFC complex with the other Rfc subunits, or failed to interact with other proteins in the pathway, e.g. PCNA. Our *E. coli* expression studies of mutant RFCs have

shown that all complexes containing a single subunit with a Lys → Glu mutation assembled into isolatable complexes (6). However, if a mutant subunit showed a profound instability in yeast, this could result in the observation of a phenotypic defect that is unrelated to the ATP binding properties of the mutant subunit. Conversely, overexpression of a mutant subunit could conceivably suppress its phenotypic defect.

The levels of the mutant subunits were determined by Western analysis. Both the mutant Rfc1R and the Rfc1E subunit were present at levels comparable with the wild type control, i.e. differences in protein levels were less than 2-fold (data not shown). Similarly, the Rfc4R and Rfc4E subunits were present at levels comparable with wild type, and those levels did not change significantly when the strains were grown in the presence of hydroxyurea (Fig. 3). Furthermore, the Rfc2R and Rfc3R subunits were also present at levels comparable with those of the respective wild type subunits (data not shown). Expression of the Rfc2E and Rfc3E subunits could not be directly measured because the mutations were lethal. The RFC2, *rfc2R*, and *rfc2E* genes were placed under control of the galactose-inducible GAL1-10 promoter cassette. Upon growth on galactose-containing medium, overproduction of Rfc2E was equivalent to that of Rfc2 or Rfc2R, indicating that the stabilities of the mutant and wild type proteins are similar (see below and Fig. 4A). The analogous result was obtained with the Rfc3E subunit (Fig. 4B). These data indicate that all mutant RFC genes were appropriately expressed. Because defects in interactions with other components in the pathway may also have phenotypic consequences, a suppression analysis was carried out.

Overexpression of PCNA Suppresses Defects in rfc4R—Because of the known physical and functional interaction between RFC and PCNA, we tested whether the observed defects were suppressed by overproduction of PCNA, as has previously been observed in the case of the *rfc1-1* and *rfc5-1* mutations (27, 28).

FIG. 3. Expression of Rfc4 mutants. Strains PY94-K, PY94-R, and PY94-E were grown in YPD or YPD containing 100 mM hydroxyurea, and extracts were prepared as described under "Experimental Procedures." The blots were probed with anti-Rfc4 antibodies. *wt*, wild type.

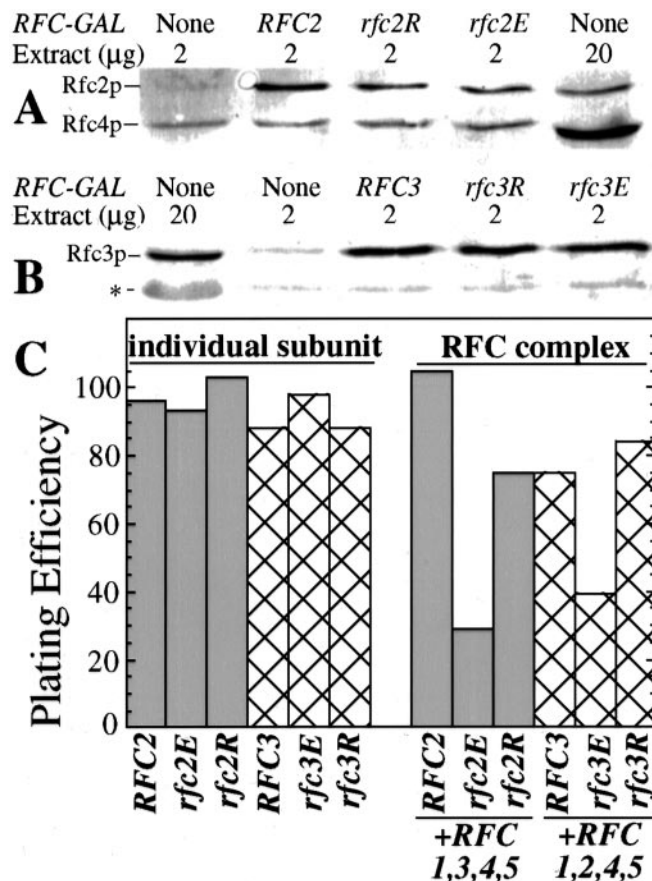
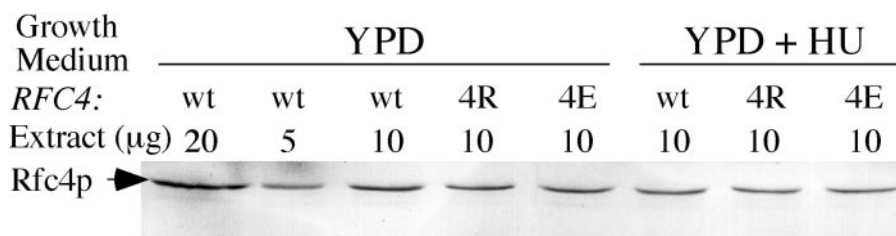


FIG. 4. Overproduction of RFC containing Rfc2E or Rfc3E is deleterious. A, strain PY73 containing pBL412 (*RFC2*), pBL412-R (*rfc2R*), pBL412-E (*rfc2E*), or empty vector (*None*) was grown overnight on selective SCGL medium (Table II). At a density of 5×10^6 cells/ml, galactose (2%) was added, and growth continued for 6 h. Extracts were made, and Western analysis was carried out as described under "Experimental Procedures." Rabbit serum raised against Rfc4p cross-reacts with the other small Rfc subunits. The serum was affinity-purified with Rfc2p expressed in *E. coli*. The affinity-purified antibodies recognized both Rfc2 and, more strongly, Rfc4, as indicated on the blot. B, growth and analysis of PY73 containing pBL413 (*RFC3*), pBL413-R (*rfc3R*), pBL413-E (*rfc3E*), or empty vector (*None*) was similar as described above. Rabbit serum raised against Rfc3p was affinity-purified with Rfc3p, expressed in *E. coli*. The diffuse band indicated with an asterisk is unrelated to Rfc subunits and was only observed in the analysis of extracts and not of purified RFC. C, plasmid-containing strains as indicated above or containing in addition either pBL424 (*RFC1*, *RFC3*, *RFC4*, and *RFC5* under *GAL1-10* control) or pBL425 (*RFC1*, *RFC2*, *RFC4*, and *RFC5* under *GAL1-10* control) were grown on selective SCGL medium and then plated on selective SCGL plates containing either 2% raffinose or 2% galactose. Plating efficiency (%) on galactose plates versus raffinose plates was determined.

The *rfc2R* and *rfc4R* strains were transformed with a multicopy plasmid carrying the *POL30* gene, which overproduces PCNA about 20-fold, and growth was monitored. Whereas growth of the *rfc2R* mutant remained unaffected, the cold sensitivity of *rfc4R* was almost completely suppressed (Fig. 2). Lethality of the *rfc2E* and *rfc3E* mutants was not suppressed by overexpression of PCNA (data not shown).

Similarly, in an *rfc5-1* strain, overexpression of *RAD24* was able to suppress both the temperature-sensitive phenotype as well as the DNA damage sensitivity exhibited in this mutant (12). The same experiments were carried out as above, but with *RAD24* on a multicopy plasmid. However, overexpression of *RAD24* in the *rfc4R* strain could not complement the cold sensitivity of the mutant (data not shown).

Viability of Double RFC Mutants—The viable single mutants were combined by appropriate crossings to determine whether RFC could tolerate the combined mutations in two of its subunits. Nine of the thirteen possible double ATP-binding motif mutants were inviable (Table III). The lethality of these double mutants may be the result of combined ATP-binding defects but could also result from cumulative defects in protein-protein interaction and complex stability. The stability and activity of double mutant RFCs was not investigated in our biochemical studies. More informative than the combined lethality was the observation that four double mutant strains, *rfc1R/2R*, *rfc1R/3R*, *rfc1R/4E*, and *rfc2R/4E*, were viable. In the viable double mutants the *rfc1-K359R* mutation is represented most often. The *rfc2R/4E* double mutant has a much more severe phenotype than the *rfc2R* strain. This mutant had a doubling time 2.1 times that of wild type. In addition, the *rfc1R/4E* as well as the *rfc2R/4E* strains were cold-sensitive for growth (Table III). None of the single or double mutants were temperature-sensitive at 37 °C (data not shown).

Mutant RFC Complexes Display a Dominant Negative Phenotype—The mutant RFC genes that were inviable in yeast were overexpressed in a wild type background (PY73) to determine whether they displayed a dominant negative phenotype. Wild type and mutant *RFC2* and *RFC3* genes were cloned into multicopy plasmids under control of the galactose-inducible *GAL1-10* promoter. Previous studies have shown that an approximately 20–100-fold overproduction of Rfc subunits was achieved upon growth on galactose (29). Plasmid-containing cells were grown under selective conditions on synthetic complete medium containing raffinose as a noninducing carbon source and then plated onto the same medium, but containing either raffinose or galactose. Overproduction of the single subunits in strain PY73 was 10–20-fold for either the wild type or mutant subunits (Fig. 4, A and B). If overexpression of a mutant subunit were deleterious, this would be evident by a decreased plating efficiency and/or poor colony growth on galactose versus raffinose plates. Surprisingly, neither of the two mutant genes with lethal mutations (*rfc2E* and *rfc3E*) showed a dominant negative phenotype when overexpressed individually (Fig. 4C). We reasoned that perhaps these mutant gene products were not efficiently incorporated into mutant RFC complexes in comparison with the wild type gene products. Therefore, we determined whether dominant negative activity could be achieved by overexpression of entire RFC complexes containing the respective mutant subunits.

When either the *rfc2E* or *rfc3E* mutant gene was overexpressed in conjunction with the appropriate four wild type genes, the plating efficiency was reduced by 60–70%. Moreover, those colonies that did appear on the galactose plates grew very poorly (data not shown). As controls, overproduction

of RFC complexes with *rfc2-K71R*, *rfc3-K59R*, or the wild type genes had little or no effect. This analysis is somewhat complicated because overproduction of wild type RFC complexes may also cause growth impairment under some conditions (29). However, as the data in Fig. 4C show, the impairment caused by overexpression of mutant complexes, specifically those containing mutations known to be inviable, was more severe, indicating that these mutant complexes are deleterious to yeast.

Sensitivity to DNA-damaging Agents—Mutant strains were tested for sensitivity to the DNA replication inhibitor hydroxyurea. The RFC mutant strains were plated on rich medium (YPDA) containing 110 mM hydroxyurea, and growth was scored after 3–4 days at 30 °C (Table III). All mutants and double mutants that harbored the *rfc2-K71R* mutation failed to grow on hydroxyurea-containing medium, indicative of a replication and/or checkpoint defect in these mutants. Surprisingly, the *rfc4R* mutant failed to grow on hydroxyurea, whereas *rfc4E* showed no defect. This was surprising because in previous investigations of ATP-binding proteins a Lys → Glu mutation invariably disrupted function much more severely than the conservative Lys → Arg mutation (25, 30–34). Indeed, this expected phenotypic pattern was observed during our studies with the *RFC1*, *RFC2*, and *RFC3* genes. To investigate whether unexpected sequence changes had occurred in these strains, the *RFC4* mutant plasmids were recovered from the yeast strains, and the mutant *RFC4* genes resequenced as well as recloned into fresh plasmid vectors. No sequence changes beyond those originally introduced were observed, and the studies with the new clones duplicated the previous results. In addition, as stated before, no significant differences in Rfc4 protein levels between the wild type and the *rfc4R* and *rfc4E* mutants were detected, indicating that the observed phenotype is inherent to the mutation and not to differences in protein levels (Fig. 3). Protein levels also remained unaffected when the cells were grown in the presence of the replication inhibitor hydroxyurea. The sensitivity to hydroxyurea exhibited by the *rfc4R* mutant can be explained by its apparent checkpoint defect (see below).

PCNA is involved in numerous DNA repair pathways (21, 35–38). Because RFC loads PCNA onto DNA, it is expected to be involved in these pathways as well. Therefore, the ATP-binding motif mutants were tested for sensitivity to UV light and MMS. Most mutants displayed a similar pattern of sensitivity to both of these agents (Table III), although the mutants were generally more sensitive to MMS than to UV. A notable exception was the *rfc1R/4E* strain that was sensitive to UV but not to MMS.

Fig. 5 shows the results of a typical time course analysis of MMS survival. For the *RFC4* mutants we again observed a reversal of the expected phenotypes; the *rfc4R* mutant was sensitive to MMS, whereas the *rfc4E* mutant was indistinguishable from wild type. In fact, a phenotype for the *rfc4-K55E* mutation could only be uncovered when it was combined in a double mutant with *rfc1-K359R* or with *rfc2-K71R*. These double mutants were more sensitive to UV and MMS than the *rfc1R* or *rfc2R* single mutants, respectively (Table III and Fig. 5).

Checkpoint Defects in RFC Mutants—In wild type strains transcription of the *RNR3* gene as well as certain other genes involved in DNA repair are up-regulated in response to DNA damage or treatment with hydroxyurea (39). Defects in the S phase checkpoint can result in a loss of induction of *RNR3* in response to damage, as observed in an *rfc5-1* or DNA polymerase epsilon mutant strain (15, 28). Mutant RFC strains containing single and double ATP-binding domain mutations were

examined for their ability to induce *RNR3* after MMS treatment, using a reporter system in which the β -galactosidase gene is placed under control of the *RNR3* promoter (39). Compared with the corresponding wild type, the *rfc2R* and *rfc4R* mutant strains were compromised in their ability to induce *RNR3* following exposure to 0.01% MMS (Fig. 6). The *rfc2R* mutant induced *RNR3* to only 15% of wild type, whereas the *rfc4R* mutant induced *RNR3* to 20% of wild type levels. These data suggest that these mutants are impaired in activation of the checkpoint in response to MMS.

Double ATP-binding motif mutants were also tested. The *rfc2R/4E* mutant, like the *rfc2R* mutant was unable to fully induce *RNR3* in response to MMS-induced damage. The *rfc1R/4E* mutant was also partially impaired in the ability to up-regulate *RNR3*. Because this double mutant showed no significant sensitivity to MMS, it was puzzling to note that this strain should be partially defective in inducing *RNR3* in response to MMS damage. However, this strain is quite sensitive to UV irradiation. Interestingly, the *rfc1R/2R* double mutant strain was able to up-regulate *RNR3*, indicating that the checkpoint defect exhibited by the *rfc2R* strain was eliminated by the additional *rfc1-K359R* mutation. However, the double mutant is still sensitive to DNA-damaging agents. These data do suggest that *RFC2* and *RFC4* have a role in cell cycle checkpoint regulation.

In a *rfc5-1* mutant strain both sensitivity to damaging agents and the checkpoint phenotypes were partially suppressed by overproduction of Rad24p (12). To determine whether a similar compensation occurred in the *rfc4R* mutant, *RAD24* was overexpressed from a multicopy plasmid in the mutant strain, and the *RNR3* induction assay was repeated. No suppression of the checkpoint defect was observed. Furthermore, the sensitivity of *rfc4R* to DNA-damaging agents remained unchanged when *RAD24* was overexpressed (results not shown). The *rfc2R* strain could not be tested for complementation of the *RNR3* induction defect because of the poor growth of cells containing both the *RNR3* induction plasmid and the *RAD24* overexpression plasmid.

RAD53 encodes a protein kinase that plays a central role in regulating the DNA damage response throughout the cell cycle (39). Rad53p is phosphorylated in a Mec1p-dependent manner and via *DUN1* leads to transcriptional induction of the DNA damage regulon, which includes *RNR3* and other genes (reviewed in Ref. 40). To confirm a possible role for *RFC2* and *RFC4* in checkpoint regulation, phosphorylation of the Rad53 protein in response to MMS was examined. In asynchronous cultures, Rad53p was phosphorylated to an approximately equal extent in both wild type and mutant strains exposed to MMS, suggesting that at least some of the DNA damage checkpoints remained intact in these cells (data not shown).

To examine damage-induced Rad53p phosphorylation during S phase, the cells were synchronized with α -factor, released, and then treated with MMS during S phase. As shown in Fig. 7, the *rfc2R* mutant that exhibited decreased *RNR3* induction is impaired in its ability to phosphorylate Rad53p. The most slowly migrating, multiply phosphorylated forms of Rad53p did not appear in the *rfc2R* mutant during the time scale of the experiment. In addition, the hypophosphorylated form of Rad53p was continuously present in the mutant, whereas in the wild type strain this form disappeared within 30 min of MMS treatment. The *rfc4R* mutant showed a similar but less dramatic defect. Similar results were obtained when the cells were irradiated with ultraviolet light (data not shown). The results for the *rfc2R/4E* double mutant were similar to those for the single *rfc2R* mutant, *i.e.* phosphorylation of Rad53p in response to damage was inhibited (data not shown).

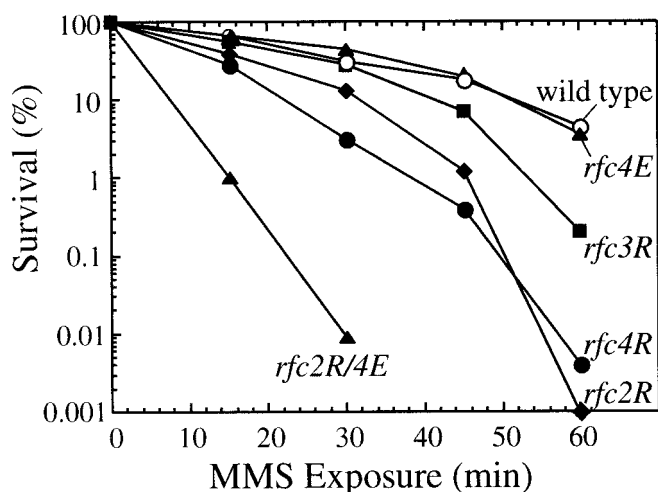


FIG. 5. **Survival of mutant strains after MMS treatment.** Cell survival was measured following treatment in 0.5% MMS for the indicated times. For details see "Experimental Procedures."

Interestingly, examination of the *rfc1R/4E* double mutant, which showed a reduced capacity for induction of *RNR3* in response to MMS (Fig. 6), did not reveal a MMS-induced defect in Rad53p phosphorylation either in asynchronous or in S phase cultures (data not shown). Therefore, the checkpoint likely remains intact. Because this double mutant is also not sensitive to MMS, the defect in *RNR3* induction upon treatment with MMS may originate from other causes than the intra-S checkpoint.

Telomere Length Maintenance—Mutations in *RFC1* have been shown to affect telomere length regulation (41). A dramatic size increase of telomeres was observed in a cold-sensitive *rfc1-5* mutant strain, where the mutation (D513N) mapped close to the ATP-binding domain. The mutant strains were tested to see whether they exhibited altered telomere length maintenance. After extended growth (>100 generations), the telomeres in the mutant strains were approximately the same length as those of wild type (results not shown). These results suggest that the telomere lengthening defect is allele-specific and not related to ATP binding.

DISCUSSION

The mutational analysis described here and in the previous paper has begun to elucidate the role of the ATP-binding motifs of the *Saccharomyces cerevisiae* Rfc subunits. Table IV summarizes the results of our genetic analysis and compares them with the biochemical analysis of the same mutants (6). There is a remarkable degree of agreement between the growth phenotype and the *in vitro* clamp loading defect, indicating that the major and essential function of RFC in the cell is that of clamp loading. This agreement is immediately apparent for the *rfc2E* and *rfc3E* mutations, which are lethal and result *in vitro* in an almost total defect in clamp loading. Similarly, the *rfc2R* mutation results in a moderate defect in clamp loading *in vitro*, and consequently the mutant strain shows severe growth defects. The *rfc3R* and *rfc4E* mutations confer a wild type growth phenotype. This does not necessarily indicate that these latter mutant complexes are as active as wild type in the cell but rather that clamp loading defects if any do not result in observable growth defects under the conditions tested. Interestingly, *in vitro* the RFC complex containing either mutation is defective for clamp loading at low ATP concentrations, but full clamp loading ability is restored at 1 mM ATP. Because in yeast neither one of these mutations confers a growth defect, it is likely that the ATP K_m defect is suppressed by the high intracellular levels of ATP of ~2 mM (42). Finally, RFC containing

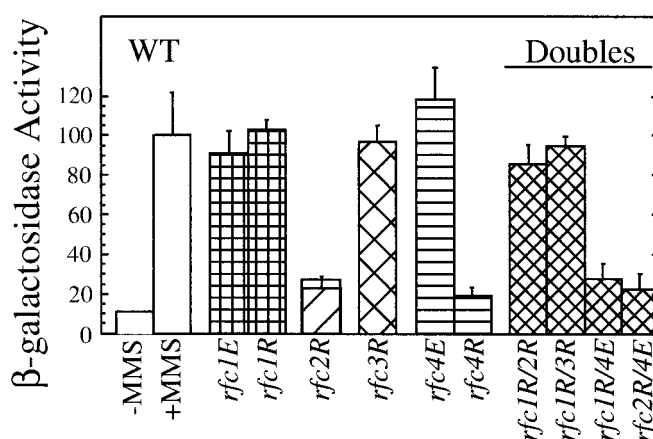


FIG. 6. **Induction of *RNR3* in response to MMS.** The cells were grown to early log phase and treated with 0.05% MMS for 5 h. The cells were broken open, and β -galactosidase activity was measured in a liquid assay. Single mutants and the corresponding wild type strains as well as the double mutants were tested. Error bars indicate standard error. Each assay in triplicate was carried out independently three to five times. Wild type (WT) levels of induction were adjusted to 100. Absolute β -galactosidase units for wild type strains after MMS induction were: *RFC1* = 124, *RFC2* = 77, *RFC3* = 79, *RFC4* = 54, *RFC1/RFC2* = 101, and *RFC3/RFC4* = 101.

the *rfc1E* mutation shows virtually no *in vitro* clamp loading defect, although there are issues with protein stability, at least when overexpressed in *E. coli*, and this mutation shows only a very minor defect in cell growth (Fig. 2).

These studies also point out the danger of using solely a genetic approach to study the role of ATP binding/hydrolysis in a given pathway and reaffirm the necessity to carry out an integrated genetic and biochemical study to assess the functional importance of ATP binding to putative ATP-binding proteins. Our genetic studies suggest that ATP binding to Rfc1 and to Rfc4 may not be important or may even be dispensable for clamp loading, because the Lys \rightarrow Glu mutation in either subunit shows little or no phenotypic defect. However, our biochemical studies show that ATP binding to Rfc4 is essential for clamp loading, whereas ATP binding to Rfc1 appears to be dispensable (6). On the other hand, both the genetic and biochemical studies indicate that ATP binding to Rfc2 and Rfc3 is essential for RFC function. The difference between mutations in the *RFC2* and *RFC3* genes on one hand and the *RFC4* gene on the other hand results from the fact that the Rfc2E or Rfc3E subunit no longer binds ATP, but the Rfc4E subunit still binds ATP, although only at very high ATP concentrations. Therefore, both the *in vivo* and *in vitro* phenotypes primarily reflect the high sensitivity of the ATP-binding domain in Rfc2 or Rfc3 and the low sensitivity of this domain in Rfc4 to mutagenesis.

Considering the lethal phenotype of the *rfc2E* mutation, we expected that overproduction of this mutant subunit in a wild type yeast strain would have a dominant negative phenotype, but a dominant negative phenotype was only observed if the remaining four (wild type) subunits were also overproduced (Fig. 4). One explanation for these observations may be that the wild type Rfc2 subunit assembles more readily into an RFC complex than the Rfc2E subunit, and therefore, despite 10–20 fold overproduction of Rfc2E, the concentration of the mutant RFC complex could be low. The deleterious phenotype observed when the four remaining wild type subunits were overproduced together with Rfc2E could then be rationalized by the accumulation of mutant complexes that are defective in clamp loading. That the Rfc2E subunit does assemble into a stable mutant RFC complex, at least when expressed in *E. coli*, follows from our previous studies (6). A parallel explanation can be offered

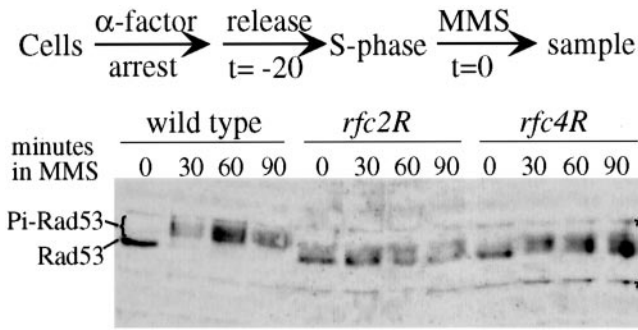


FIG. 7. Phosphorylation of Rad53p. The cells were treated with α -factor and released from arrest for 20 min at 30 °C prior to addition of MMS. Time points were every 30 min from the time of MMS addition. The positions of Rad53p and the series of phosphorylated Rad53p species (P_i-Rad53p) are indicated. The asterisks indicate the positions of bands unrelated to Rad53p.

TABLE IV
Biochemical and genetic defects in RFC mutants

The data for the biochemical properties are from Ref. 6. Minor, >30% activity or ++± for growth; moderate, 5–30% activity; severe, 1–5% activity or + for growth; inactive, <1% activity; *K_m*, full or indicated activity at 1 mM ATP but defective at 10 μM ATP.

RFC gene	Mutation	Clamp loading defect	Growth defect	Other defects
<i>RFC1</i>	Lys → Arg	–	none	none
	Lys → Gly	minor	minor	none
<i>RFC2</i>	Lys → Arg	moderate + <i>K_m</i>	severe	checkpoint
	Lys → Glu	inactive	lethal	
<i>RFC3</i>	Lys → Arg	minor + <i>K_m</i>	none	repair
	Lys → Glu	severe + <i>K_m</i>	lethal	
<i>RFC4</i>	Lys → Arg	–	minor	checkpoint
	Lys → Glu	<i>K_m</i>	none	none

for the mutant Rfc3E subunit, overproduction of which has a phenotype similar to that of Rfc2E.

This study did not provide much new information about the role of ATP in Rfc1 function. Although only minor defects were seen in the *RFC1* mutants, this does not mean that the consensus ATP-binding motif in Rfc1 is not required for another function of RFC, which was not assayed in this study. Alternatively, ATP binding to Rfc1 may play a regulatory role in the interconversion of RFC to alternative clamp loading complexes containing a Rfc2–5 core and an alternative large subunit, e.g. Rad24 (43).

The study of the *RFC4* mutants have presented an interesting deviation from the accepted rule that conservative Lys → Arg mutations show less severe phenotypes than the charge reversal Lys → Glu mutations. In the case of *RFC4*, this pattern was reversed. *In vivo*, the *rfc4-K55R* mutant had mild growth defects, particularly at 13 °C, which were partially suppressed by overexpression of PCNA. Control experiments showed equivalent expression of the wild type and mutant Rfc4 proteins. A checkpoint defect was also detected in *rfc4R* (Figs. 6 and 7). All of these phenotypes were absent in the *rfc4E* mutant. In addition, two viable double mutants with *rfc4-K55E*, i.e. *rfc1R/4E* and *rfc2R/4E*, could be isolated, but no double mutants with *rfc4-K55R* were viable. The conserved lysine residue in the Walker A motif of ATP-binding proteins has been a favorite target for mutational studies. In general, mutation of the conserved lysine in the Walker A domain of ATP-binding proteins results in only slight alterations in the P loop structure as determined by x-ray crystallography and other methods (44–46). Therefore, biochemical or genetic defects associated with mutations in this residue have in general been ascribed to defects in ATP binding and/or hydrolysis

rather than to defects in protein folding, protein stability, or association with other subunits in a complex. Folding and/or interaction defects can often be suppressed by overproduction of an interacting protein in the pathway. Remarkably, the cold sensitivity of the *rfc4R* mutant but not of the *rfc2R* mutant was suppressed by overproduction of PCNA, consistent with the interpretation that protein-protein interactions are affected in the *rfc4R* mutant, perhaps through instability of the P loop in the ATP-binding domain.

In addition to the role of RFC in DNA replication, this study provides further evidence implicating *RFC2* in checkpoint control and for the first time suggests a role for *RFC4* in this process. Between budding and fission yeast, all of the small subunits have now been demonstrated to be involved in checkpoint regulation. *RFC5* was the first RFC subunit to be identified in this role (11, 28). The *rfc5-1* mutant exhibits defects in checkpoints that respond to DNA replication block or DNA damage in S phase. Rfc5p was later found to interact with the checkpoint protein Rad24p (12). Since then, *RFC2* was also shown to be involved in checkpoint control in both budding and fission yeast (10, 13). Although this study did not implicate *RFC3* in checkpoint control, recent work suggests a checkpoint function for the *S. pombe* homolog (14). All of these genetic data are consistent with a model in which the Rfc2–5 core forms a complex with Rad24 to function in response to DNA damage during the S phase (43). The observation of a checkpoint defect in the *rfc2R* mutant suggests a requirement for ATP binding and/or hydrolysis during checkpoint function.

In conclusion, an integrated genetic and biochemical analysis of the ATP-binding domains of four Rfc subunits indicates that ATP binding to the Rfc2, Rfc3, and Rfc4 subunits of RFC is required for clamp loading, but ATP binding to Rfc1 is dispensable. Moreover, no or only marginal defects were detected in the *RFC1* mutants. However, this study was limited to phenotypic studies related to cell growth, damage sensitivity, and checkpoint function. It may well be possible that defects exist in other pathways such as recombination and chromosome segregation. Further studies are required to elucidate the role of the ATP-binding domain in the Rfc1 subunit.

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