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DNA polymerase δ, RFC and PCNA are required for repair synthesis of large looped heteroduplexes in *Saccharomyces cerevisiae*

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ABSTRACT

Small looped mispairs are corrected by DNA mismatch repair (MMR). In addition, a distinct process called large loop repair (LLR) corrects loops up to several hundred nucleotides in extracts of bacteria, yeast or human cells. Although LLR activity can be readily demonstrated, there has been little progress in identifying its protein components. This study identified some of the yeast proteins responsible for DNA repair synthesis during LLR. Polyclonal antisera to either Pol31 or Pol32 subunits of polymerase δ efficiently inhibited LLR in extracts by blocking repair just prior to gap filling. Gap filling was inhibited regardless of whether the loop was retained or removed. These experiments suggest polymerase δ is uniquely required in yeast extracts for LLR-associated synthesis. Similar results were obtained with antisera to the clamp loader proteins Rfc3 and Rfc4, and to PCNA, i.e. LLR was inhibited just prior to gap filling for both loop removal and loop retention. Thus PCNA and RFC seem to act in LLR only during repair synthesis, in contrast to their roles at both pre- and post-excision steps of MMR. These biochemical experiments support the idea that yeast polymerase δ, RFC and PCNA are required for large loop DNA repair synthesis.

INTRODUCTION

DNA looped heteroduplexes (loops) arise *in vivo* as synthetic errors within microsatellites (1–4), minisatellites (3), or between tandemly repeated sequences (5). Loops are mutagenic precursors to insertion or deletion mutations, depending on whether the newly replicated or parental strand, respectively, contains the loop. The ability to correct loops prior to a subsequent DNA synthetic event helps avoid mutations. When cells lack such correction activity, insertion and deletion mutants occur at increased frequencies (3,6–10). For example, it is thought that small insertions and deletions in important tumor suppressor genes contribute to hereditary nonpolyposis colon cancer when mismatch repair (MMR) is defective (11). Loops are also formed during recombination between alleles that differ in length, and the correction of these recombination intermediates contributes to gene conversion (12). Recombination studies in yeast and mammalian cells indicate that loops up to several kilobases undergo efficient repair (12–17).

The repair of loops is complex. Depending on loop size, they can be excellent substrates for MMR. Small loops, from 1 to ~8 nt, undergo very efficient MMR (3,18–22). However, repair efficiency by this pathway decreases to very low levels as the loop size reaches ~16 nt in eukaryotes (3,23). Thus, MMR function cannot explain repair of loops >16 nt, nor does MMR correct all molecules with loops between ~8 and 16 nt (19,22–26). Instead, several activities are reported to repair large DNA loops (13–16,23,25–33). These activities differ in efficiency, genetic requirements and response to secondary structure within the loop.

One system that we call large loop repair (LLR) corrects loops up to several hundred nucleotides in size. There is indirect but compelling evidence that LLR may be quite similar in mammalian cells, in yeast and in bacteria. First, robust LLR has been observed in several laboratories using different approaches, such as *in vivo* assays (26–28) and biochemical assays using cell-free extracts (22,25,26,29,32–34). Second, while loops of ~8–16 nt are moderately good substrates for LLR (19,22,23,25), larger loops up to several hundred nucleotides are corrected even more efficiently (25–29,32–34). The upper size limit for efficient repair has not been firmly established. Third, LLR is independent of MMR and nucleotide excision repair, as mutant cell lines lacking these repair pathways retain LLR activity (22,23,25,29,32). Fourth, the presence of a nick in loop-containing substrates stimulates repair on the nicked strand (26,29,32–35). However, some studies also report loop removal on the continuous strand of nicked molecules and in covalently closed substrates (25,26,32, 33,35). Either a nick is not absolutely required in all cases, or spurious nicking promotes LLR. Fifth, LLR can repair looped substrates to either of two products, depending on the location of the loop and a nick (25–27,29,32). In one instance, the looped strand is used as a template for DNA

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repair synthesis, resulting in a product that retains the sequence present in the loop. Alternatively, the looped strand is removed, and repair synthesis then leads to the shorter product. In substrate molecules where loop retention and loop removal can be assayed simultaneously, both outcomes have been observed *in vivo* and *in vitro*, although loop removal is usually more frequent (25,27,32,33). It remains to be determined whether loop removal and loop retention are two distinct repair activities, or if they represent alternative outcomes of the same repair pathway.

What is the role of DNA repair synthesis during correction of large loops? Examination of *in vitro* LLR under conditions where DNA repair synthesis is blocked (by omitting dNTPs, for example) showed that single-stranded gaps are formed (25,29,32). Aside from the loop itself, gaps are usually ~100–200 nt, even over a range of loop sizes (25,29,32), although shorter gaps are seen in some circumstances (32). When substrates contain a loop and a nick on opposite strands, gaps of similar sizes are observed in yeast extracts, indicating that both loop removal and loop retention occur by an excised intermediate (25). Both loop retention and loop removal therefore require DNA repair synthesis to fill the gaps. What polymerase(s) might be responsible? DNA polymerase δ (Pol δ) is a versatile enzyme used in a number of DNA repair pathways such as base excision repair (36,37) and MMR (38), in addition to its important role in replication (39). Pol δ usually requires the sliding clamp proliferating cell nuclear antigen (PCNA) for optimal processivity, and the replication factor C (RFC) clamp loader to assist the trimeric, ring-shaped PCNA molecule in encircling the DNA. Pol δ, PCNA and RFC are therefore logical candidates for the repair synthesis step of LLR. Only indirect evidence is available to support this hypothesis, however. Two groups (29,32) identified an aphidicolin-sensitive polymerase for LLR in human cell extracts. Pol δ is sensitive to aphidicolin, but so are other DNA polymerases, leaving open the question of which polymerase is involved in LLR. The mutation spectra in some replication mutants show increased frequencies of large insertion or deletion mutations, suggestive of an LLR defect. Yeast *pol32*Δ mutants, lacking the non-essential Pol32 subunit of Pol δ, do not show increased mutation rates (40,41). However, *pol32*Δ mutants show an unusual accumulation of 8–237 bp insertions flanked by direct repeats, and of 27–46 bp deletions with flanking direct repeats, both of which are rare in wild-type cells (41). Some yeast PCNA mutants (*pol3-103*, *pol30-126*) also accumulate high levels of large deletions or insertions (42), and at least one mutant allele of *rfc1* leads to increased mutations of >14 bp in poly(GT) tracts (43). These phenotypes are consistent with a possible defect in LLR. However, these phenotypes do not distinguish between the possibilities that altered protein activities generate more loops (and thus perhaps overwhelm LLR), or that these mutants have reduced ability to correct loops, or that perhaps these mutants affect both loop formation and repair.

The purpose of this study was to determine biochemically whether Pol δ, RFC and PCNA are needed for DNA repair synthesis during LLR. We used antibody inhibition experiments to examine LLR in yeast cell-free extracts. The results are consistent with the idea that Pol δ, PCNA and RFC are essential components for DNA repair synthesis during LLR for both loop retention and loop removal. These findings provide the first identification of LLR components, and add to the impressive list of activities to which Pol δ and its associated factors contribute.

MATERIALS AND METHODS

Reagents and enzymes

Standard reagents, including molecular biology grade CsCl, were obtained from Sigma. Hydroxyapatite resin was a product of BioRad. All restriction enzymes were obtained from New England Biolabs or Stratagene. Exonuclease V was from U.S. Biochemicals. Enzymatic reactions were performed as recommended by the manufacturers.

Heteroduplex preparations

LLR substrates were created as described in detail previously (25,26). Briefly, the C_{27} and V_{27} molecules used for *in vitro* repair were created by annealing DNA strands from f1 phage derivatives that differ by a 27 bp substitution. The resulting heteroduplex molecule is therefore completely complementary over its 6.4 kb length aside from the 27 nt loop. A site-specific nick located 114 bp 5′ to the loop resulted from cleavage of original double-stranded phage DNA preps with Sau96I, followed by annealing to a covalently closed single-stranded circle.

Nuclear extract preparation

Nuclear extracts of strain DY6 (*MAT***a** *ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407*; from B. Jones, Carnegie-Mellon University via T. Hsieh, Duke University) were prepared as previously described (25). Briefly, yeast cells grown to mid-log phase are treated with zymolyase to create spheroplasts, then lysed with a homogenizer to retain intact nuclei. Following enrichment of the nuclei by differential centrifugation, they are exposed to 0.2 M NaCl to allow extraction of nuclear proteins, which are subsequently concentrated by ammonium sulfate precipitation and dialyzed into buffer C (20 mM HEPES–KOH, pH 7.6, 10 mM $MgSO_4$, 10 mM EGTA, 5 mM DTT, 20% glycerol, 2 μM pepstatin A, 0.6 μM leupeptin, 2 μg/ml chymostatin, 2 mM benzamidine and 1 mM PMSF). Protein concentrations were determined using BioRad Protein Assay.

Large loop repair assays

LLR was monitored by previously described methods (25,26,29). Briefly, extracts were exposed to polyclonal antisera or to control pre-immune sera for 60 min on ice with occasional gentle mixing. For no antibody control reactions, buffer C was added instead. Heteroduplex DNA (100–150 ng, corresponding to 24–36 fmol of DNA molecules) and 10× repair cocktail (yielding final assay concentrations of 20 mM HEPES–KOH, pH 7.6, 1 mM glutathione, 1.5 mM ATP, 0.1 mM of each dNTP, 0.05 mg/ml BSA) were added. Reaction volumes were 10–15 μl unless otherwise stated. Repair occurred for 45–60 min at 30°C, then 30 μl of 25 mM EDTA was added to quench the reaction. Negative control reactions had EDTA added prior to DNA. DNA was subsequently purified by phenol extraction and ethanol precipitation, then treated for 60 min at 37°C with 4 U EcoRI plus 6 U ClaI to monitor

loop removal, or with 5 U NheI plus 6 U ClaI to score loop retention. In some cases, DNA prior to restriction cleavage was treated with 2 U Klenow fragment plus 33 μM dNTPs for 30 min at 37°C. Following restriction, the products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized and quantitated with a Kodak EDAS 290 system. LLR activity is expressed as amount of DNA converted to product (3.3 + 3.1 kb bands) divided by the total DNA in the lane (6.4 + 3.3 + 3.1 kb bands) × 100%. Negative control lanes were used to assess the background level of cutting, and presumably reflect small amounts of homoduplex DNA in some substrate preparations.

Antisera

Rabbit polyclonal antisera were raised previously against purified yeast proteins Pol31, Pol32, Rfc3, Rfc4 and PCNA. Antiserum to Rfa1 was the generous gift of Steven Brill (Rutgers University).

RESULTS

Substrates for monitoring LLR catalyzed by yeast nuclear extracts

Figure 1 shows two heteroduplex substrates, C_{27} and V_{27}, containing 27 nt loops. Previous work (25,29,32) demonstrated that correction of these loops occurs by LLR, not by MMR or nucleotide excision repair, due to the size of the loop. Yeast LLR (25,26) and possibly human LLR (32) correct heterologies by two modes. In one mode, the loop is removed regardless of the presence or absence of a nick. Alternatively, the second LLR mode targets the strand containing a pre-existing nick for excision and resynthesis, regardless of which strand contains the loop. Both modes are examined in the experiments described later. The looped substrates contain a nick 114 bp 5′ to the loop. Figure 1A shows that loop-stimulated and nick-stimulated repair on C_{27} both lead to the same EcoRI-sensitive product, because the loop and the nick are on the same strand. This feature makes C_{27} a relatively robust substrate for *in vitro* assays. V_{27}, where the loop and nick reside on opposite strands, provides additional information about LLR because two different products are seen. Loop-stimulated activity is detected as EcoRI sensitivity, but nick-stimulated repair leads to an NheI-sensitive product (Figure 1B; only the last repair product shown in Figure 1 is diagnostic of an inserted sequence, whereas the other three cases represent deletion outcomes.). LLR of V_{27} is therefore partitioned into two products, each of which is diagnostic for a distinct mode of LLR.

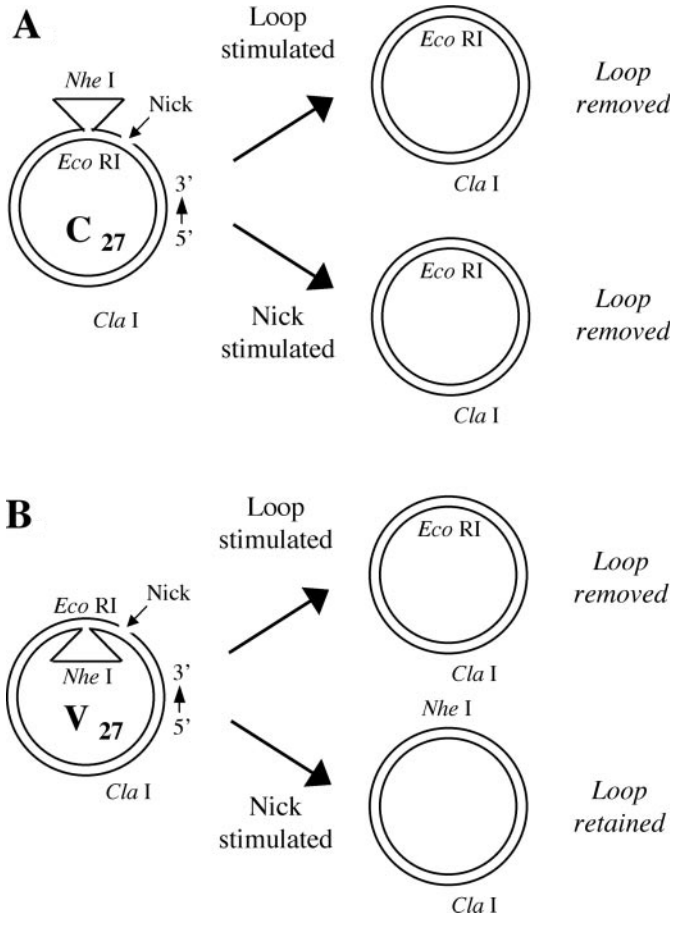

Figure 1. Large loop substrates and repair products for *in vitro* assays. (**A**) The heteroduplex substrate contains a 27 nt loop on the complementary (C) strand. The strand with the inserted sequence includes an NheI site, whereas the opposite strand contains a site for EcoRI. The loop renders the heteroduplex resistant to both NheI and EcoRI. Hence double digests of ClaI with either enzyme result in a full-length (6.4 kb) linear product. A site-specific nick was present on the C strand 114 bp 5′ to the loop. If repair on C_{27} is targeted to the loop strand (top arrow), the DNA becomes sensitive to EcoRI. If repair is targeted to the discontinuous strand (bottom arrow), the product is also EcoRI sensitive. Thus both modes of repair on C_{27} contribute to the accumulation of EcoRI-sensitive product. (**B**) The V_{27} substrate has the loop on the viral (V) strand, and the sequence of the loop is the complement to that found for the C_{27} loop. Otherwise the two heteroduplexes are identical. Repair of V_{27} targeted by the loop results in EcoRI sensitivity. In contrast, nick-stimulated LLR leads to an NheI-sensitive product that retains the loop sequence. Only the last repair product shown is diagnostic of an inserted sequence, whereas the other three cases represent deletion outcomes. The use of V_{27} allows differentiation of LLR that is loop-directed from nick-stimulated.

Antisera to Pol δ inhibit LLR *in vitro*

The role of Pol δ in other DNA repair pathways suggested that it might also be involved in LLR. Pol31 and Pol32 are auxiliary subunits that form a tight complex with the Pol3 catalytic polypeptide. Thus yeast Pol δ is purified as the heterotrimeric Pol3–Pol31–Pol32 complex (40). To test the role of Pol δ in LLR, yeast nuclear extracts were incubated with either pre-immune or immune polyclonal antisera to Pol31 or Pol32, and then assayed for LLR. The Pol31 and Pol32 subunits were chosen for analysis, rather than Pol3, due to the availability of high-titer antisera. The residual LLR activity, relative to a no antiserum control, is shown in Figure 2. In the presence of Pol31 or Pol32 antisera, LLR activity was reduced to 10–19% of the untreated control in a dose-dependent manner. In contrast, the pre-immune sera did not reduce the LLR activity of the extract (104–146%). Clearly, these polyclonal antisera to the Pol δ subunits, Pol31 and Pol32, are potent inhibitors of LLR in yeast extracts.

Since antisera to both Pol31 and Pol32 yielded similar suppression of LLR, the most likely explanation is that inhibition of Pol δ results in loss of overall LLR. If so, LLR in the presence of the antisera might still excise the loop, but be

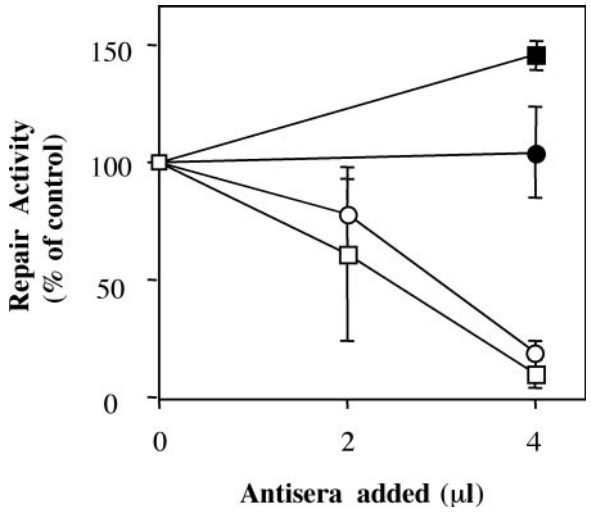

Figure 2. Inhibition of LLR by antisera to Pol31 or Pol32. Yeast nuclear extract (4 µl, containing 12 µg of total protein) was mixed with the indicated volumes of rabbit polyclonal antisera or pre-immune control antisera. Buffer C was added as necessary to generate a total volume of 8 µl. A control reaction without antisera was included. The mixture was incubated on ice for 1 h with occasional gentle mixing. Next, 2 µl of a mixture containing equal volumes of C_{27} heteroduplex DNA (100 ng) plus 10× repair cocktail were added, and the reactions were incubated for 45 min at 30°C. Reactions were then quenched with EDTA and processed as described in Materials and Methods. LLR was quantitated and normalized to the no antiserum control, which in these experiments gave an average value of 20% repair. Filled squares, pre-immune Pol31 serum; filled circles, pre-immune Pol 32 serum; open circles, immune Pol32 serum; open squares, immune Pol31 serum. Error bars indicate the range of values observed in two independent experiments.

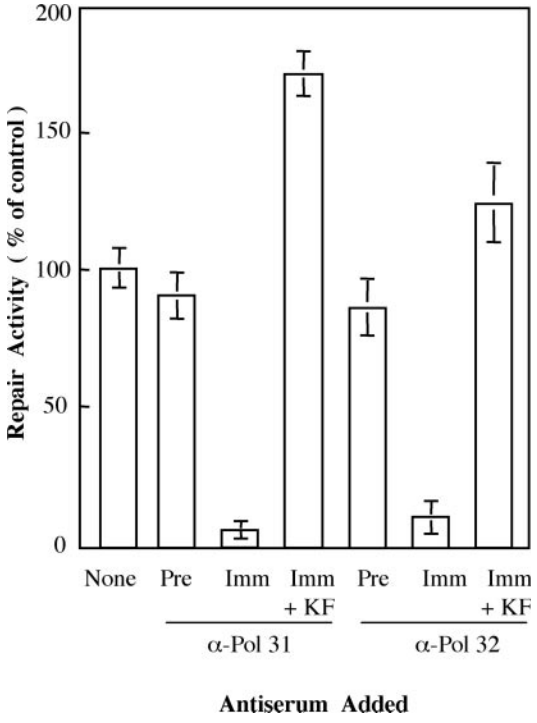

Figure 3. Rescue of LLR by addition of Klenow fragment. Yeast nuclear extracts (4 µl) were mixed with 4 µl of either buffer C (no antibody control) or pre-immune sera to Pol31 or Pol32. A second set of reactions contained 8 µl extract plus 8 µl antisera to Pol31 or Pol32. The substrate was C_{27}. Incubations and DNA recoveries were as described in the legend to Figure 2 and in Materials and Methods. Before restriction digests, the samples incubated with immune sera were split in half and one sample of each was incubated for 30 min at 37°C with 33 µM dNTPs plus 2 U Klenow fragment. All samples were then scored with EcoRI and ClaI as described in Materials and Methods. Results are normalized to the no antibody control, which in these experiments yielded 18% repair. Bar heights indicate the mean for two to three experiments and error bars indicate the range. The polymerase addition results are labeled as '+KF'.

unable to perform repair synthesis (25,29,32). This predicts that the DNA intermediates from reactions with inhibiting antisera contain single-stranded gaps, which could then be filled in by exogenous polymerases. To test this prediction, the experiment was repeated. A portion of the recovered DNA (after phenol extraction and ethanol precipitation) was treated with Klenow fragment in the presence of dNTPs, and then assayed for sensitivity to EcoRI to monitor restoration of repaired, double-stranded DNA. Klenow fragment was chosen due to its ready availability and because it can repair gaps efficiently without the need for accessory proteins. Figure 3 shows the results. Compared to control reactions without antisera, the pre-immune controls showed little inhibition (86–90% activity remained) whereas the experiments with immune anti-Pol31 or anti-Pol32 sera were strongly inhibited (6–11% activity). This confirms the findings from Figure 2. When the DNA from the inhibited reactions was treated with Klenow fragment (Figure 3), EcoRI sensitivity was restored to 123–169% of the control value. Therefore, exogenous polymerase activity rescued the defect created by the anti-Pol δ antisera. In contrast, addition of Klenow fragment to an EDTA-quenched control was without effect, as expected. The results from Figures 2 and 3 are consistent with the idea that Pol δ is the only polymerase in these extracts capable of performing this gap-filling function. Furthermore, it suggests that Pol δ acts solely at the DNA repair synthesis step, and does not participate in the steps leading up to and including excision. The ability of Klenow fragment to generate LLR levels higher than the control (Figure 3) suggests Pol δ might be partially limiting in our extracts. In other words, does addition of purified Pol δ to an uninhibited extract generate higher levels of LLR? We found that LLR levels could be enhanced ~25% by the addition of 25 ng of purified Pol δ or by including 1–2 units of Klenow fragment, but that addition of 25 ng of RFC or 50 ng of PCNA did not detectably increase LLR activity (data not shown). Thus Pol δ seems to be partially limiting for LLR in these cell-free preparations.

The experiments described above used the C_{27} substrate, in which the loop and the nick are on the same DNA strand (Figure 1), to maximize the sensitivity of detection. It was also useful to examine the effects of anti-Pol δ antisera on the V_{27} substrate that has the loop and nick on opposite strands. The interest in this experiment stems in part from the suggestion by Li and colleagues (32) that large loop removal from covalently closed strands by human cell extracts may involve very small gaps and therefore DNA synthesis might be limited. If this were the case in yeast, then perhaps another polymerase could substitute for Pol δ during loop removal. This model predicts that large loop removal from the covalently closed strand of V_{27} would be insensitive to anti-Pol δ antisera. To test this possibility, we examined the effect of the antisera on V_{27} repair to either the loop retention product or to the loop removal product. Figure 4 shows very similar results for both repair outcomes. The pre-immune

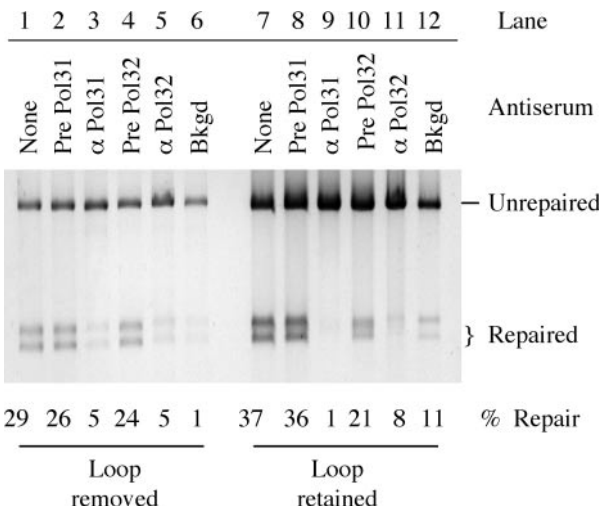

Figure 4. Anti-Pol δ antisera effects on repair of the V_{27} heteroduplex substrate. Nuclear extract (2.3 µl) was incubated with 9 µl pre-immune or immune antisera plus 14.2 µl Buffer C. A no antibody control contained extract plus 23.2 µl of Buffer C. After 60 min incubation on ice, 1.5 µl V_{27} DNA (300 ng) plus 3 µl of 10× repair cocktail were added, and the mixture incubated for 45 min at 30°C. After DNA purification by phenol extraction and ethanol precipitation, each sample was split 25:75. The smaller aliquot was tested for loop removal by EcoRI plus ClaI (lanes 1–6), and the larger aliquot examined for loop retention by NheI plus ClaI (lanes 7–12). More DNA is necessary for clear detection of the loop retention reaction because it occurs at a lower level than loop removal (25). The Figure shows an ethidium bromide stained gel, displayed as the photonegative for ease of viewing. Lanes 1 and 7, no antibody control; lanes 2 and 8, pre-immune Pol31 control serum; lanes 3 and 9, anti-Pol31 antiserum; lanes 4 and 10, pre-immune Pol32 control serum; lanes 5 and 11, anti-Pol32 antiserum; lanes 6 and 12, EDTA-quenched negative control to show background cutting of the substrate (the background in lane 12 was spuriously high in this experiment; background levels of 1–5% are typical). Similar results were seen in three other repetitions of this experiment.

controls for both Pol31 and Pol32 gave modest or no inhibition (LLR at 57–97% of control value), whereas the immune antisera were effective at blocking both loop removal and loop retention reactions (3–22% of control). Thus loop repair in yeast extracts requires Pol δ for gap-filling regardless of whether the loop is removed or retained.

As a test for the specificity of the antisera used in the inhibition studies, we asked whether purified Pol δ could reverse the inhibition. Extract was incubated with a sub-saturating amount of Pol31 antiserum. This antiserum level was specifically chosen to partially inhibit the reaction, so that changes in LLR activity could be detected upon subsequent addition of purified Pol δ. Figure 5A shows that the Pol31 antiserum reduced LLR activity by ~40% under these conditions, relative to the no antiserum control (lane 2 versus lane 1). When 25 ng of purified Pol δ was added at the end of the antiserum incubation, LLR activity rose to approximately the same level as seen in the control (lane 3 versus lane 1). Figure 5B shows the reproducibility of the results for this experiment. The add-back of pure Pol δ reversed the inhibition of the antiserum.

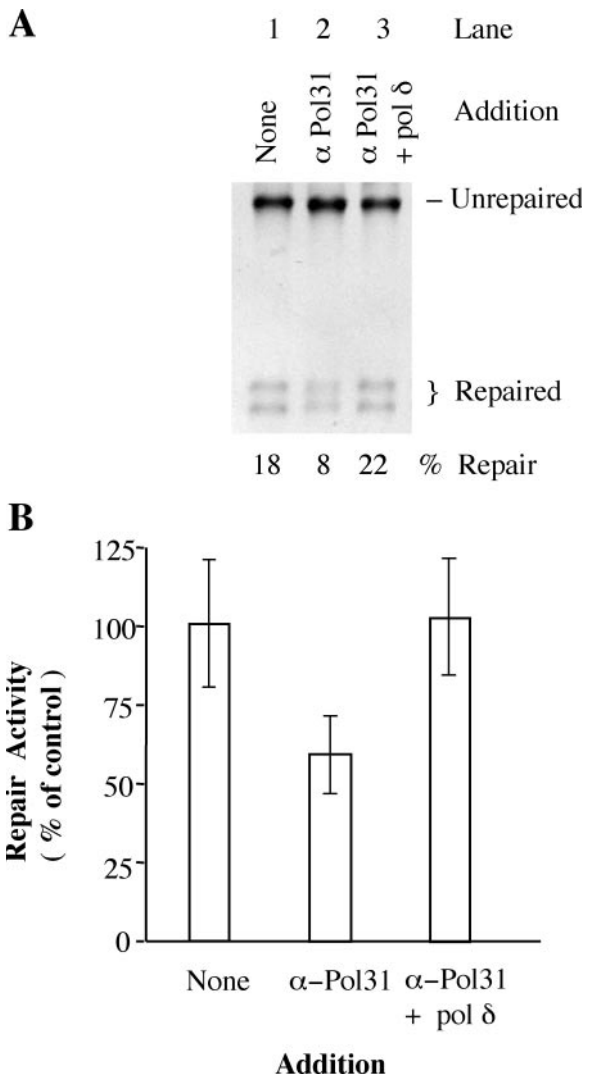

Figure 5. Add-back of Pol δ reverses the inhibitory effect of Pol31 antiserum. (**A**) Nuclear extract and Pol31 antisera (1.0 µl each) were incubated with 6 µl Buffer C. For the no antiserum control reaction, the volume of Buffer C was 7 µl. After 60 min incubation on ice, 1 µl (25 ng) purified Pol δ was added as indicated. V_{27} DNA (2 µl, 100 ng) plus 2 µl of 10× repair cocktail were immediately added to all tubes, followed by incubation for 60 min at 30°C. LLR activity was measured as loop removal (measured by cleavage with EcoRI plus ClaI). V_{27} was used in this experiment due to availability of the substrate, and loop removal was measured to facilitate detection. Lane 1, no antibody control; lane 2, anti-Pol31 antiserum only; lane 3, anti-Pol31 antiserum plus purified Pol δ. (**B**) Compilation of results from three independent repetitions of the experiment. Repair activity is normalized to the no antiserum control, which yielded 18–27% repair in these experiments. Error bars represent ±1 SD.

Antisera to Rfc3, Rfc4 or PCNA also inhibit LLR *in vitro*

Optimal Pol δ activity in replication and repair usually requires the sliding clamp PCNA and the clamp loader, RFC. If Pol δ participation in LLR has similar requirements, inhibition of PCNA or RFC should reduce LLR. Inhibition studies were therefore performed using antisera to Rfc3 and Rfc4 (subunits of RFC) and to PCNA. These antisera are known to inhibit activity *in vitro* for RFC (44) and PCNA (P. Burgers, unpublished observations), respectively. Figure 6 shows that pre-immune serum slightly stimulated LLR activity with the C_{27} substrate to 108–111% of control level. In contrast, antisera to Rfc3, Rfc4 or PCNA reduced LLR activity to 9–30% of control values. The effect was dependent on the amount of antiserum added in all three

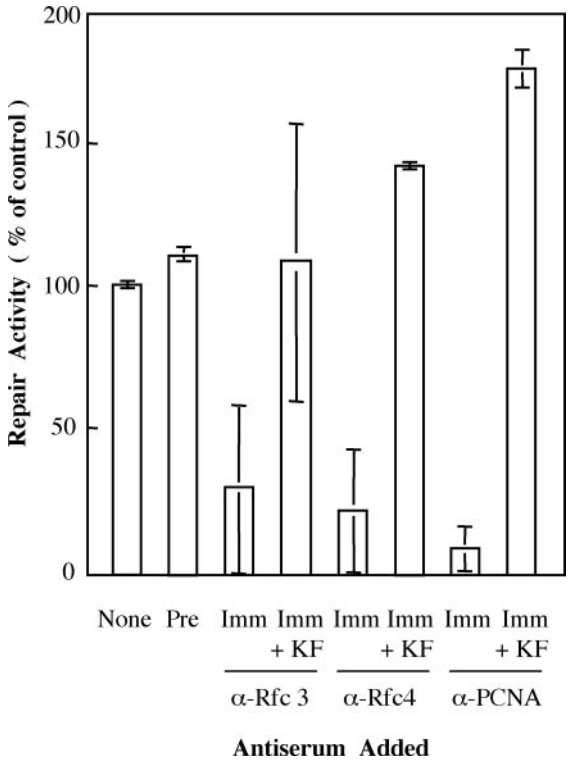

Figure 6. Inhibition of LLR by antisera to Rfc3, Rfc4 or PCNA, and rescue by addition of Klenow fragment. Yeast nuclear extracts (2 µl) were mixed with 2 µl of either buffer C (no antibody control) or pre-immune sera. A second set of reactions contained 4 µl extract plus 4–8 µl antisera to Rfc3, Rfc4 or PCNA, or Pol31 pre-immune serum. The substrate was C_{27}. Incubations and DNA recoveries were as described in the legend to Figure 2 and in Materials and Methods. Samples incubated with immune sera were split in half and processed as described in the legend to Figure 3. All samples were then scored for EcoRI sensitivity. Results are normalized to the no antibody control, which in these experiments yielded 37% repair. Bar heights indicate the mean for two experiments and error bars indicate the range. The Klenow fragment addition results are labeled as '+KF'.

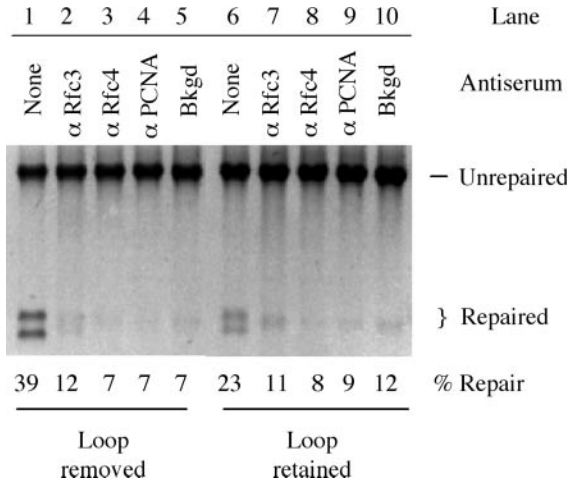

Figure 7. Anti-RFC and anti-PCNA antisera effects on repair of the V_{27} heteroduplex substrate. Nuclear extract (4.5 µl) was incubated with 12 µl immune antisera plus 6 µl Buffer C. A no antibody control contained extract plus 18 µl of Buffer C. After 60 min incubation on ice, 4.5 µl V_{27} DNA (300 ng) plus 3 µl of 10× repair cocktail were added, and the mixture incubated for 60 min at 30°C. After DNA purification by phenol extraction and ethanol precipitation, the sample was split 33:67. The smaller aliquot was tested for loop removal by EcoRI plus ClaI, and the larger aliquot was treated with NheI plus ClaI to score loop retention. The Figure shows an ethidium bromide stained gel, displayed as the photonegative. Lanes 1 and 6, no antibody control; lanes 2 and 7, anti-Rfc3 antiserum; lanes 3 and 8, anti-Rfc4 antiserum; lanes 4 and 9, anti-PCNA antiserum; lanes 5 and 10, EDTA-quenched negative control to show background cutting of the substrate. Similar results were seen in two other repetitions of this experiment.

cases (data not shown). We note that the range of values for the anti-Rfc3 antisera was larger than for the other antisera, but we have no clear understanding of this observation. Nonetheless, the role of RFC and PCNA in LLR is supported by the inhibitory effect of all three antisera.

We also sought to determine whether RFC and PCNA are important in LLR only for post-excision loop repair synthesis or if earlier, pre-excision steps in LLR also require these proteins. This question is based in part on the complex role for PCNA and RFC during MMR (45,46). While PCNA and RFC are unquestionably required for DNA repair synthesis following excision of the mispair, in some substrates these proteins also play an important role in stimulating the excision step (45,46). Thus PCNA and RFC can act both pre- and post-excision for repair of mismatches. To determine when PCNA and/or RFC function during LLR, we performed LLR reactions in the presence of antisera, recovered the DNA by phenol extraction and ethanol precipitation, and then treated with Klenow fragment plus dNTPs. As described earlier, recovery of EcoRI-sensitive material following Klenow fragment fill-in synthesis indicates that LLR excision but not resynthesis had occurred. Figure 6 shows that restriction enzyme sensitivity was restored by Klenow fragment ('+KF') for the samples treated with antisera to Rfc3, Rfc4 and PCNA. The level of EcoRI-sensitive material following Klenow fragment activity was 108–174% that of the no antibody control reaction (Figure 6). Like the anti-Pol δ experiment shown earlier (Figure 3), the enhanced level of repair product is consistent with the idea that Pol δ–dependent resynthesis activity is partially limiting in the yeast nuclear extract. The major finding of this experiment, however, is that both PCNA and RFC are required after excision, during the repair synthesis step of LLR.

The V_{27} substrate was utilized to examine the role of RFC and PCNA in both loop removal and loop retention modes of LLR. If both repair outcomes have similar requirements for RFC and PCNA, then similar levels of inhibition should be seen with antiserum inhibition. Figure 7 shows the outcome of the experiment. Exposure of the extract to α-Rfc3, α-Rfc4 or α-PCNA antisera reduced loop removal to 18–31% of control activity, similar to background (compare lanes 2–4 with background in lane 5). When loop retention was tested, inhibition by the antisera was again observed to 35–48% of control, and similar to background (compare lanes 7–9 with lane 10). These results indicate that RFC and PCNA, like Pol δ (Figure 4), are important for resynthesis of gaps associated with both outcomes of LLR, namely loop removal and loop retention.

We also tested antiserum to the RFA subunit Rfa1, to see if single-strand binding activity is important for LLR. Also, RFA is active in MMR (47–49), and RFA helps Pol δ in most synthetic reactions (50). The anti-Rfa1 antiserum gave mixed results when assayed for loop removal on V_{27} and C_{27} substrates (data not shown). Partial inhibition was seen in some experiments while no inhibition was detectable in others (data not shown). Probably, the RFA complex is present

in such high abundance that the antiserum cannot neutralize enough molecules to reduce repair. Alternatively, RFA might play a limited role in LLR resynthesis. We favor the former explanation, since single-stranded gaps of ~100–200 nt are formed during LLR (25,29,32), and RFA would probably stimulate repair synthesis of gaps in this size range.

LLR inhibition cannot be explained by salt effects or non-specific inhibitors present in the antisera. Dose-dependent inhibition of LLR activity was seen with five different antisera (to Pol31, Pol32, Rfc3, Rfc4 and PCNA). In contrast, several pre-immune control sera failed to inhibit repair activity, and partial to no LLR inhibition was seen with anti-Rfa1 antiserum as noted earlier. If salt or other components of the antisera were causing inhibition, we would expect loss of LLR activity in all cases, but this was not observed.

DISCUSSION

The antibody inhibition experiments indicate Pol δ is required in extracts for yeast LLR. This conclusion is supported by the following observations: (i) Immune antisera for both Pol31 and Pol32 yielded identical results. (ii) Two pre-immune sera gave little or no effect. (iii) The immune antisera blocked LLR at a step after excision but prior to repair synthesis. (iv) Both loop retention and loop removal were similarly inhibited by the two antisera. (v) Add-back of purified Pol δ reversed inhibition. Together these results provide clear evidence that Pol δ is required for yeast LLR *in vitro*. Furthermore, repair synthesis is similar on both the nicked and closed strands, judged by the results in Figure 4 and by the gap sizes seen when dNTPs are omitted (25). These observations suggest that repair synthesis is reasonably extensive for both nick-stimulated and loop-directed LLR in yeast—about 100–200 nt—compared to the limited synthesis reported for loop removal in human cell extracts (32). Our antibody inhibition experiments cannot exclude the possibility, however, the fact that *in vivo* another DNA polymerase might substitute for Pol δ but that this other polymerase is present in our extracts at low levels or in some inhibited form, thereby leaving *in vitro* repair dependent solely on Pol δ. One line of pre-existing evidence indicates that Pol δ is the most likely polymerase in LLR. Huang *et al* (41) found that the *can1* mutation spectrum in *pol32*Δ mutants shows a significant shift from mainly single-base alterations and frameshifts towards large insertions and deletions. While the overall mutation rate in *pol32*Δ mutants is at or slightly below wild-type levels (40,41), clearly there is an increased accumulation of large insertions and deletions. This genetic evidence is consistent with our biochemical results.

Like Pol δ, RFC and PCNA also show activity in LLR in yeast extracts. Incubation of extracts with appropriate antisera led to strong inhibition of repair that was attributable to failure to resynthesize gaps formed during LLR. Thus, the role of RFC and PCNA seems limited to DNA repair synthesis in this system. This finding is in contrast to their multifunctional roles in MMR (45,46). This difference is consistent with the observation that MMR and LLR in human cell extracts yield qualitatively different intermediates when repair synthesis is blocked (29). Our findings also show that both possible outcomes of LLR—loop removal and loop retention—require RFC and PCNA in yeast extracts. Genetic evidence for an *in vivo* role of PCNA and RFC in LLR is consistent with our conclusions, since yeast strains harboring *pol3-103* or *pol30-126* mutations accumulate high levels of large deletions or insertions (42), and an *rfc1::Tn3* mutant allele showed an excess number of >14 bp mutations in a poly(GT) tract (43).

Our biochemical studies provide the first indication of a direct role for Pol δ, RFC and PCNA in LLR repair synthesis. Since loop removal and loop retention are both dependent on these proteins, the simplest interpretation is that a single repair pathway in yeast leads to both products. Alternatively, it is possible that two distinct sets of proteins initially act in loop removal and loop retention, but that both pathways utilize common resynthesis machinery. As far as their role *in vivo*, it remains to be determined whether Pol δ, RFC and PCNA act only during loop repair synthesis, or if they also help prevent formation of the loops in the first place. Nonetheless, the results of this study identify the first protein components of LLR, and they add another DNA metabolic activity to the list for DNA Pol δ, RFC and PCNA.

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