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RecA Interacts with Klenow and Enhances Fidelity of DNA Synthesis *in vitro*

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Abstract

To understand the molecular basis of RecA-mediated DNA-repair, we tested the replicative fidelity of the large fragment of Pol I (Klenow) in RecA-DNA complexes *in vitro*. Klenow synthesis was error-prone in naked DNA substrates but essentially error-free in RecA coated complexes. *Escherischia coli* SSB, causes no such improvement in Klenow fidelity. RecA filaments promote better exonucleolytic proofreading by Klenow than on naked DNA substrates at select sites when replication is "stalled" due to a missing dNTP. Addition of RecA to pyrene sulfonylchloride-labeled Klenow resulted in a specific increase in steadystate fluorescence anisotropy and a concomitant decrease in fluorescence lifetime. These observations suggest the possibility of a direct interaction between RecA and Klenow even in the absence of DNA which may mediate the observed improvement in Klenow fidelity.

Introduction

RecA protein is central to homologous genetic recombination and recombinational DNA repair (Cox, 1993). While recombination confers genetic diversity, recombinational repair, on the other hand, is critical for the very survival of the cell. An important feature of recombinational repair is DNA replication which follows RecA mediated pairing (Asai *et al*., 1994). It is believed that RecA-DNA complexes need to functionally interact with replicating enzymes during repair events (Cao and Kogoma, 1995). Suggestive evidence that RecA protein may also be involved in Pol I mediated repair stems from the observations that mutants lacking *recA* and DNA pol I^{ts} are not viable and show extensive DNA degradation after the transfer to 42 °C, the non-permissive temperature (Gross *et al*., 1971; Monk and Kinross, 1972; Monk *et al*., 1973). However, the mechanism of this lethality has not been elucidated.

To investigate the nature of the interaction between RecA and Pol I at the molecular level, we tested the replicative fidelity of the large fragment of Pol I (Klenow fragment) in RecA-DNA complexes *in vitro*. RecA-ssDNA nucleoprotein complexes are active only in the presence of ATP and are highly conserved structurally in all procaryotic and eucaryotic organisms (Radding, 1993). A complementary primer annealed to the RecA coated template served as a prototype of a recombination complex that was tested for DNA extension by Klenow. The results described here demonstrate that Klenow extension shows enhanced fidelity in RecA-DNA complexes as compared to that in naked DNA substrates. Fluorescent labeled Klenow revealed a direct and specific interaction with RecA even in the absence of DNA, suggesting that protein-protein interaction may mediate the functional collaboration between RecA and Klenow.

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Results

RecA-DNA Complexes Enhance Fidelity of Klenow Synthesis

A 5'-end-labeled 33-mer primer was annealed to an 83-mer template either by RecA catalyzed reaction or by thermal annealing (Figure 1). Following annealing, the buffer composition of the thermal reaction mix was made identical to that of the RecA reaction mix. To stay close to the native reaction conditions of Pol I, we wanted to perform primer extension in the presence of all four dNTP's and analyze the products directly. As a novel approach, we tried to assess errors in the products by directly sequencing the strand using the Maxam-Gilbert chemical sequencing method. We hoped that a direct comparison of the chemical sequencing ladder of primer extension from substrates in RecA-filament *vis-a-vis* that of extension from naked DNA under identical conditions should reveal the differences in Pol I fidelity, if any. DNA samples were extracted from primer extension reactions following deproteinization and were subjected to chemical sequencing. G and A+G cleavages were compared for Klenow extension products obtained from both thermal and RecA reactions. Scrutiny of differences in sequence-ladders between RecA and thermal reactions in primer and primer-extension regions revealed an important result: Sequencing ladders for RecA and thermal reactions were identical in primer regions whereas those corresponding to primer-extension regions were not. Extension products showed discernible levels of error specifically in lanes corresponding to thermal samples. Several G-misincorporations, showed up in extensions of residues 3 to 11 (bracket a, lane 1, Figure 2A). Bands corresponding to such misincorporations, although faint, were present only in lanes corresponding to thermal samples. In the same extension reactions, three error bands showed up in the A+G cleavage lane (bracket b, lane 3, Figure 2A). Again, the RecA lane was devoid of these error bands (bracket b, compare lane 3 with 4, Figure 2A). It is to be noted that the high intensity bands seen in thermal reactions (Figure 2A, lanes 1,3 just below the arrow where the arrow indicates the position of the first nucleotide added) is due to leftover primer from incomplete annealing which is clear from the gel electrophoretic analyses of

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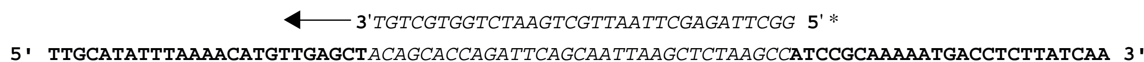
Substrate A



Substrate B

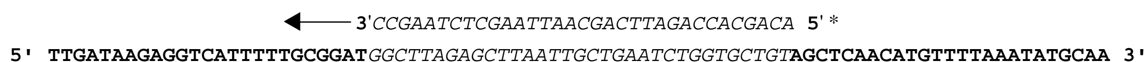


Figure 1. Two Template-Primer Substrates used in the Experiments
The sequence in italics is the pairing region. The asterix at the 5' ends denotes ^{32}P label, and the arrows indicate the direction of primer extension.

annealed samples in native conditions (Figure 3A). Native gel analysis of overloaded annealed samples revealed that RecA-mediated annealing was essentially complete whereas that by thermal annealing was somewhat incomplete with respect to labeled primer. The standard chemistry of cleavages envisaged in Maxam-Gilbert reactions shortens the chain of DNA from its original length (Maxam and Gilbert, 1980). The leftover unannealed primer in the thermal sample fortuitously comigrates with one of such Maxam-Gilbert cleavage products of the replicated strand. For a stricter quantitative comparison, error bands were normalized with the Phosphor-Imager scans of correct-bands (bracket b, lane 3 versus 4, Figure 2A). This essentially ruled out any effects arising from minor differences in sample-loads in different lanes. The scan from the RecA sample was essentially free of any error-band whereas that from the thermally annealed sample revealed three distinct peaks (Figure 2B). The data suggested that Klenow error levels could be detected by the chemical sequencing approach followed by appropriate normalization using Phosphor-Imager scans. However, this approach is essentially a qualitative and comparative one and is not amenable to quantify the errors. The error-bands in thermal samples were uncovered only after Maxam-Gilbert modification reactions of the samples. Extension products following piperidine cleavages without any prior base modifications showed no bands in thermal as well as RecA lanes (Figure 3B). This control not only ruled out any artifact of differential cleavages by piperidine in RecA versus thermal samples, but also that of any differential incomplete extensions (Figure 3B). The controls demonstrated that the entire labeled primer in RecA and most of the same in the thermal samples was extended into full length 58-mer strands. The only other band seen was that of a small fraction of starting 33-mer primer that was left unannealed in thermal sample just as it was seen in native gel analysis descibed above (Figure 3A and 3B). No other cleavage products were seen in this control indicating that the sequencing ladders of RecA *versus* thermal samples in Figure 2A were genuine products of Maxam-Gilbert sequence-specific modification. The same small fraction of 33-mer primer that was unannealed was detected in thermal lanes following Maxam-Gilbert sequencing also (Figure 2A). Although sequencing of extended products

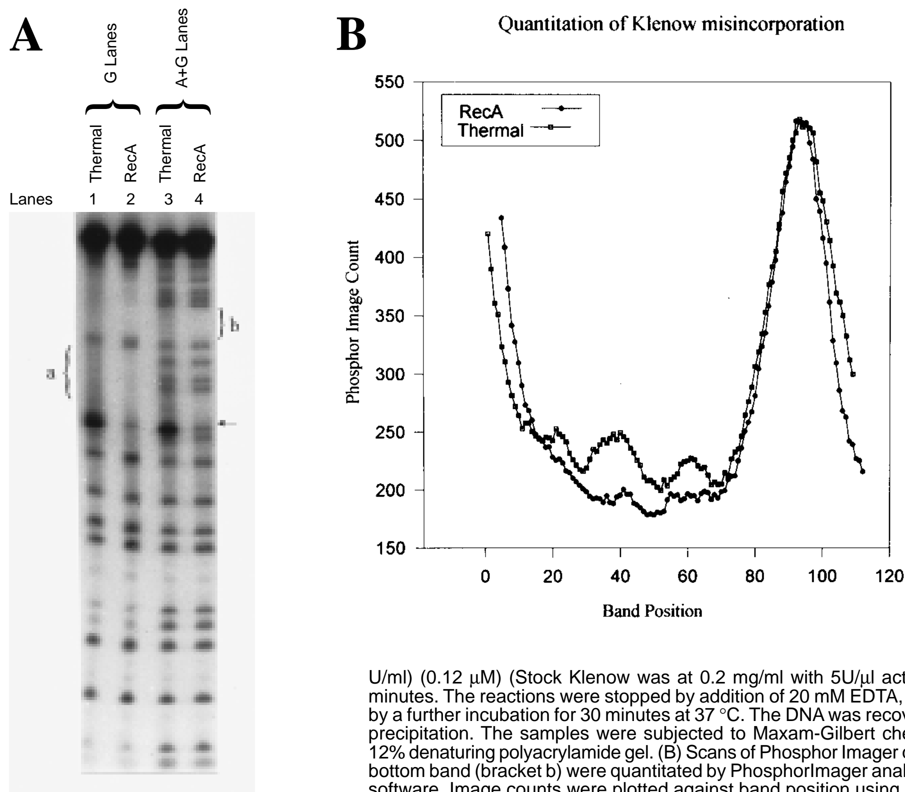
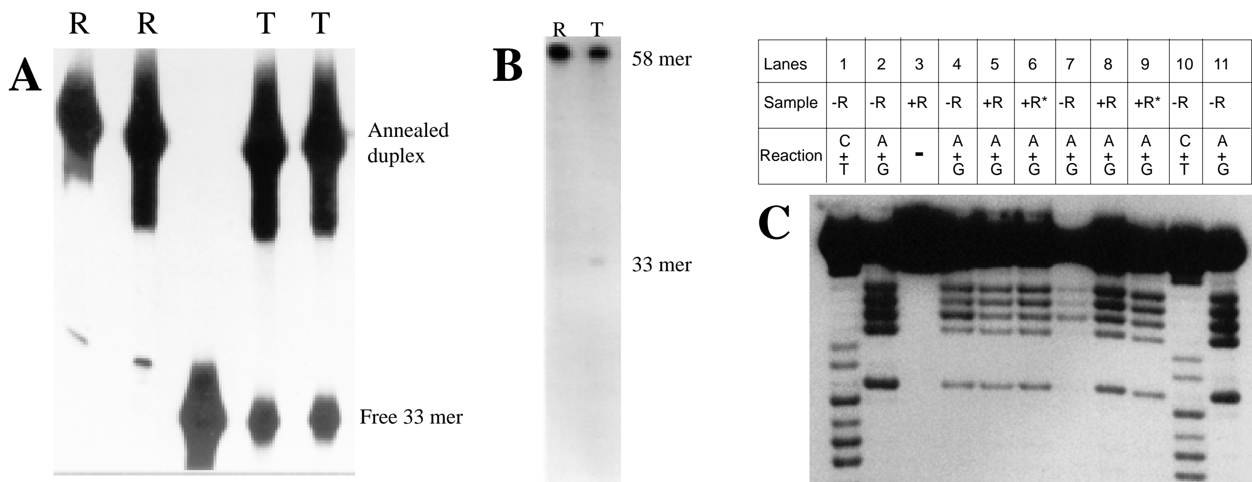


Figure 2. Maxam-Gilbert Chemical Sequencing of Klenow Extension Products
(A) Sequencing gel. Arrow refers to the position of first nucleotide synthesized. Template 83-mer oligonucleotide (10 µM) (Substrate A; Figure 1) was incubated with RecA (3 µM) in a reaction containing 33 mM Tris-HCl (pH 7.5), 1.2 mM magnesium acetate, 2 mM DTT, 1.2 mM ATP, 8 mM creatine phosphate, creatine phosphokinase (10U/ml) and BSA (100 µg/ml) for 15 minutes at 37 °C followed by pairing with 5'-^{32}P-labeled 33-mer primer oligonucleotide (3 µM) for an additional 45 minutes at 37 °C. Thermal annealing was done by heating the oligonucleotides at the same concentrations as in the RecA reaction above, at 85 °C for 5 minutes in 33 mM Tris-HCl (pH 7.5), 1.2 mM magnesium acetate followed by slow cooling to room temperature. The buffer composition of the thermal annealing sample was made identical to that of the RecA reaction by adding appropriate amounts of DTT, ATP, creatine phosphate, creatine phosphokinase and BSA. Primer extensions were performed by addition of 10mM magnesium acetate, all four dNTP's (100 µM each) and Klenow enzyme (200 U/ml) (0.12 µM) (Stock Klenow was at 0.2 mg/ml with 5U/µl activity), followed by incubation at 37 °C for 30 minutes. The reactions were stopped by addition of 20 mM EDTA, 0.2% SDS and 1 mg/ml proteinase K followed by a further incubation for 30 minutes at 37 °C. The DNA was recovered by phenol extraction followed by ethanol precipitation. The samples were subjected to Maxam-Gilbert chemical sequencing followed by analysis on a 12% denaturing polyacrylamide gel. (B) Scans of Phosphor Imager quantitations. The error-bands and the adjacent bottom band (bracket b) were quantitated by PhosphorImager analysis (Molecular Dynamics) using Image Quant software. Image counts were plotted against band position using Sigma Plot.



Lanes	1	2	3	4	5	6	7	8	9	10	11
Sample	-R	-R	+R	-R	+R	+R*	-R	+R	+R*	-R	-R
Reaction	C + T	A + G	-	A + G	A + G	A + G	A + G	A + G	A + G	C + T	A + G

Figure 3. (A) Comparison of RecA versus thermal annealing reactions on native polyacrylamide gel. Annealing reactions were performed between template strand and ^{32}P-labeled 33-mer primer oligonucleotide as per the conditions described above (see Legend, Figure 2A). Samples were deproteinized by proteinase K treatment as above and analyzed in duplicates (of 25 µl each) on a 10% polyacrylamide gel under native conditions in cold room. The gel was dried and autoradiographed. (R: RecA annealed; T: Thermally annealed). (B) Analysis of Klenow extended samples following piperidine cleavage without prior Maxam-Gilbert chemical modification. Annealing reactions between template strand and ^{32}P-labeled 33-mer primer oligonucleotide followed by Klenow extensions and deproteinizations were carried out as described in Figure 2A, legend. Samples were directly subjected to piperidine cleavage as for the Maxam-Gilbert sequencing protocol (Maxam and Gilbert, 1980), but without any prior chemical modification of bases. Samples were analyzed on a 12% denaturing polyacrylamide gel (R: RecA annealed; T: Thermally annealed). (C) Comparison of Maxam-Gilbert chemical sequencing specificity of naked DNA with the same in RecA-complex. A 33-mer ss-DNA (5'-GCTACAAACG-AATGGATCCTCATTAAAGCAGTT-3') (16 µM) (100 µl) was incubated with either RecA (8 µM) (R) or heat-killed RecA (R*) (8 µM) or equivalent volume of RecA-buffer (30 mM sodium cacodylate, pH 8.0) (-R) in 30 mM sodium cacodylate (pH 8.0), 2 mM magnesium acetate and 1 mM ATP-γ-S at 37 °C for 15 min. The samples were split into two sets, one of which was treated with 0.1% dimethyl sulphate (DMS) (lanes 4-6) and the other with 0.2% DMS (lanes 7-9) for 2 min at 37 °C. All the six samples and additional naked DNA controls (lanes 1, 2, 10 and 11) were processed further according to standard Maxam-Gilbert sequencing procedures followed by analysis on a 15% denaturing polyacrylamide gel and autoradiography. The bottommost bands in lanes 1 and 2 represent 5C and 4A residues respectively of the given 33-mer. Lane 3 sample is from RecA-ssDNA complex that was piperidine treated without prior Maxam-Gilbert chemical modifications.

was performed in RecA-samples after removal of RecA by proteinase-K followed by phenol extraction and ethanol precipitation, and sequencing ladders for RecA versus thermal samples were identical in primer regions (Figure 2A), we were still concerned whether any residual RecA that remained in the RecA sample contributed to the observed differences in sequencing ladders between RecA and thermal sample lanes in Figure 2A. We decided to test for such a potential sequencing artifact by directly comparing Maxam-Gilbert sequencing specificity of a naked 33-mer oligonucleotide of known sequence with the same when it was covered by RecA protein. In fact, such a control offers an “exaggerated version” for the above mentioned hypothetical sequencing artifact, since it has a full complement of RecA-protein in it (both functional and non-functional, see below). RecA binding was stabilized by using a nonhydrolyzable ATP analogue, namely ATP-γ-S (Kowalczykowski and Eggleston, 1994). Binding was confirmed by gel-shift and protein-based filter binding assays which showed that more than 90% of the labeled oligonucleotide was in RecA-coated complexes (data not shown). We probed such RecA-coated complexes and naked oligonucleotides under identical conditions of Maxam-Gilbert sequencing reactions that were specific for A+G residues. The specificity of sequencing was identical for both samples (Figure 3C). At two different concentrations of dimethyl sulphate that specifically modifies A+G residues, the RecA-coated oligonucleotide behaved identically to naked DNA (compare lanes 4 and 7 with 5 and 8 respectively in Figure 3C). Heat-killed RecA served as another control to prove that functionally active as well as inactive forms of RecA give rise to no aberrations in Maxam-Gilbert sequencing specificity (compare lanes 6 and 9 with 5 and 8 respectively in Figure 3C). The specificities observed were identical in RecA-oligomer complexes, DNA incubated with heat-inactivated RecA and naked DNA alone. These controls ruled out any unknown sequencing artifacts associated with RecA samples and assured us of the authenticity of sequencing differences observed between Klenow extensions of RecA versus thermal samples in Figure 2A as true fidelity differences.

If the fidelity improvement by RecA were true, it should depend on the ATP since all known biochemical functions of RecA critically require ATP-bound form of RecA which

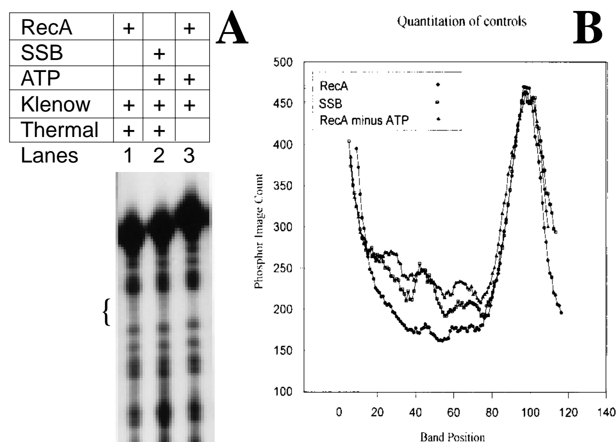


Figure 4. Chemical Sequencing of Klenow Extensions from Control Reactions
(A) Sequencing gel. After thermal annealing, the sample was made identical to the buffer-composition of the RecA reaction from which ATP was omitted (Substrate A, Figure 2, legend). RecA (3 µM) was added to this and incubated for 15 minutes at 37 °C. In another control, *E. coli* SSB (1 µM) was added to a thermal sample that was made identical to the buffer composition of the RecA reaction, followed by incubation at 37 °C for 15 minutes. Both these controls (lanes 1,2 respectively) and a RecA-annealed sample (lane 3) were extended by Klenow, followed by sequence analysis as described in the Figure 2 legend. (B) Scans of Phosphor-Imager quantitations as in Figure 2 legend.

is popularly known as "active RecA" (Kowalczykowski and Eggleston, 1994). In order to test the specificity of fidelity improvement by RecA, we analysed the primer extension products from thermal reactions that contained RecA in the absence of ATP, an essential nucleotide cofactor for RecA activities (Kowalczykowski and Eggleston, 1994). The omission of ATP in the RecA reaction failed to improve Klenow fidelity and showed the same level of error-prone synthesis as that seen in thermal reactions (Figure 4A). Error levels quantitated by Phosphor-Imager scans of RecA reaction that lacked ATP were essentially similar to those of thermal reactions (Figure 4B, compare with Figure 2B). As an additional control, the primer extension reaction was done with thermally annealed substrate in the presence of another single-stranded DNA binding protein, *E. coli* SSB. The conditions of binding were chosen such that SSB bound to all the substrate DNA as revealed by gel retardation assay (data not shown). Addition of SSB did not inhibit the primer extension reaction and the synthesis was error prone (lane 2, Figure 4A). A Phosphor-Imager scan quantitatively demonstrated that primer extension in the presence of SSB was more error-prone than that of a RecA reaction containing ATP. However, it was less than that of either a thermal reaction or a RecA reaction in the absence of ATP (Figure 4B, compare with Figure 2B). The above results qualitatively demonstrated the error prone nature of Klenow synthesis on naked DNA substrates while the same substrates in RecA-filaments yielded error free products by Klenow under otherwise identical conditions. It is possible that the rate of polymerization could affect the fidelity, since the polymerase had to access the primer from within a nucleoprotein filament. We analysed the rate of synthesis by Klenow in the following experiments.

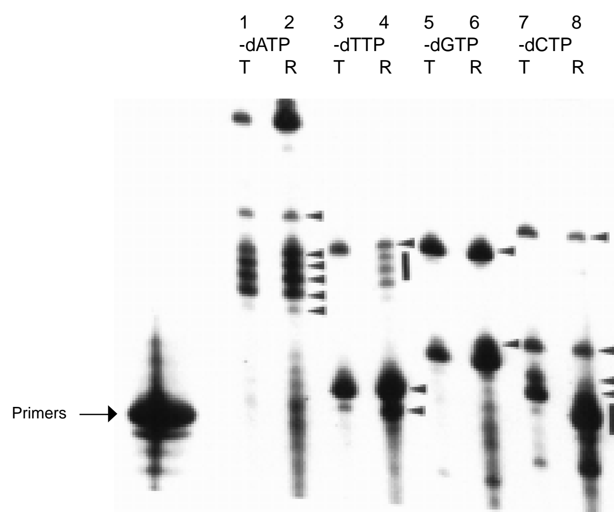


Figure 5. Comparison of 3' to 5' Proofreading in "Stalled" Complexes
Primer extensions were done with Substrate B (Figure 1) as per the conditions described in the Figure 2 legend with the following changes. In each reaction, a dNTP was omitted. Following the arrest of Klenow reactions by EDTA/SDS/Proteinase K treatment, an aliquot of 5 µl from each reaction was added to an equal volume of sequencing-gel loading buffer and analyzed on a 12% denaturing polyacrylamide gel. (T: Thermal; R: RecA; Arrowheads: Stalled products; Vertical bars: 3' to 5' degraded products).

The Rate of Klenow Extensions in RecA-DNA Complexes

Circular single-stranded chimeric M13 genome (M13G*ori*1) was annealed with a complementary 83-mer oligonucleotide, (template in substrate B, Figure 1) either by thermal annealing or by RecA mediated pairing. Efficiency of annealing was assessed by using 5' end-labeled 83-mer oligonucleotide followed by electrophoretic analysis on a native agarose gel (data not shown but was very similar to that shown in Figure 3A). However, for Klenow rate measurements, annealing reactions were done with unlabeled 83-mer. In both thermal and RecA reactions, about 19-20 nucleotides were incorporated per second per each substrate molecule, which is comparable to the reported values (Kornberg and Baker, 1992). Other controls with SSB and RecA in the absence of ATP gave similar rates of incorporation as summarised in Table 1. There was no appreciable difference in the rates of synthesis by Klenow in these different conditions. To gain an insight into how RecA reaction improves fidelity of DNA synthesis, we compared exonucleolytic editing in "stalled" complexes.

Exonucleolytic Proofreading in RecA Versus Thermal Reactions

Primer extensions were studied in the absence of a dNTP using substrate B (see Figure 1). Four reactions, each with a missing dNTP were compared between RecA and thermal pairings. As mentioned earlier, all thermal reactions also contained ATP to match with the conditions of RecA

Table 1. Polymerisation Rate of Klenow Expressed as nucleotides/sec/template

Reactions	Rate
Thermal	20
RecA	19
RecA minus ATP	11
SSB	15

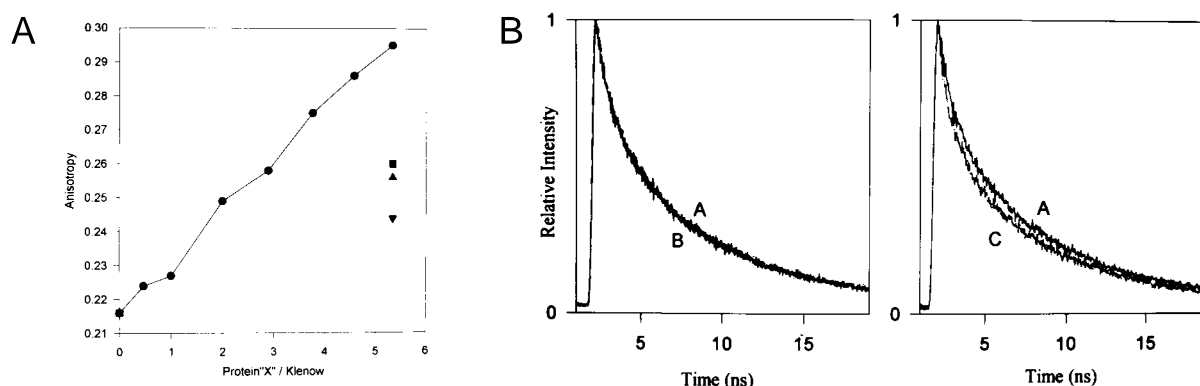


Figure 6. Physical interaction between RecA and Klenow as shown by fluorescence anisotropy analyses. (A) Fluorescence anisotropy analysis: PySCl-conjugated Klenow (1.5 μM) was excited at 350 nm in 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgSO_4$ and emission was monitored at 382 nm. To the same Klenow sample, increasing molar excesses of RecA (●) were added and fluorescence anisotropy (r_{ss}) monitored. Other parallel controls contained 5.3 molar excess of RecA that was inactivated by crosslinking (■) , BSA (▲), and lysozyme (▼). (B) Fluorescence intensity decay: The excitation and emission wavelengths were 357 nm and 382 nm respectively in the same buffer as in (A) and at molar ratio of 5.3 (protein-X to Klenow). Klenow: A; Klenow + BSA or Klenow + lysozyme: B; Klenow+RecA: C. Results of analysis of decay kinetics are given in Table 2.

reactions that included ATP as an essential cofactor. As expected, most primer extensions stalled at those sites that needed missing dNTP (arrow heads in Figure 5). In some complexes, reactions stalled at –1 positions (bottom most arrow-head in lanes 3,4; top most arrow-heads in lanes 5-8 in Figure 5). The relative intensities of stalled products were essentially similar in RecA versus thermal reactions. However, closer comparison revealed an important difference: At some sites of stalling, the products were trimmed down by a further 2-4 nucleotides specifically in RecA samples and not in thermal samples (vertical bars in lanes 4 and 8, Figure 5). Presumably these shorter products arose from higher exonucleolytic 3' to 5' processing of stalled complexes in RecA reactions which occurred at select sites in –dTTP and –dCTP reactions. The implications of such an observation are discussed later.

Klenow and RecA Interact with Each Other

In view of the fact that both Klenow and RecA bind to DNA, any test we perform to assess a direct interaction between them in the presence of DNA is more difficult to interpret. Therefore, we decided to test whether these proteins interact with each other even in the absence of DNA. A molecule rotates in solution more slowly when it is a part of a complex than when it is alone. Due to this difference in rotation, a fluorophore labeled molecule exhibits higher fluorescence anisotropy when it is a part of a complex than when it is alone. It is therefore possible to exploit this difference in fluorescence anisotropy to assay molecular interaction (Kwok *et al.*, 1994; Lundblad *et al.*, 1995; Heyduk *et al.*, 1997). In contrast to gel or immunoprecipitation assays, fluorescence anisotropy permits true equilibrium analyses of weak as well as strong interactions.

We carried out both steady state as well as time resolved fluorescence measurement using Klenow covalently attached to a fluorescent probe. Pyrene sulfonylchloride (PySCl), an amine selective agent, was chemically linked to Klenow such that specific activity of Klenow was unaffected following modification (data not shown). PySCl served as a sensitive molecular probe to report any interactions that ensued with Klenow. Klenow-Pyr showed enhanced fluorescence anisotropy (r_{ss}) as a function of increasing concentration of RecA (Figure 6A). Fluorescence anisotropy, r_{ss}, reached 0.295 from 0.216 upon the addition of RecA at 5.3 fold molar excess to Klenow (Figure 6A). These changes in anisotropy are significant and have earlier been used to demonstrate specific molecular interactions and their equilibrium constants (Heyduk and Lee, 1990; Heyduk *et al.*, 1993; Perez-Howard *et al.*, 1995; Pyles and Lee, 1996). At the same molar excess, arbitrary proteins such as BSA or lysozyme increased Klenow anisotropy to about 0.256 and 0.244 respectively (Table 2 and Figure 6A). This partial increase might be due to a weak and nonspecific interaction with Klenow which was further exemplified by another control described below. Glutaraldehyde crosslinking leads to an efficient intermolecular crosslinking of protein molecules and in proteins such as RecA leads to complete inactivation of RecA. As expected, such a reaction efficiently crosslinked all RecA molecules into high molecular weight complexes that were soluble and had lost all DNA-pairing activity of RecA (see Experimental Procedures). In the presence of such functionally inactive RecA at 5.3 fold molar excess (with respect to RecA monomer) to Klenow, the increase in r_{ss} was significantly less when compared to that with native RecA (Figure 6A). This behaviour was similar to the situation with other nonspecific binders. This control also ruled out the argument that higher increase in anisotropy by RecA was due to its aggregated state as compared to that of BSA or lysozyme. This is because glutaraldehyde crosslinking that further accentuates RecA aggregation did not lead to any further increase in anisotropy over that seen with native RecA but rather showed a reduction in the value of r_{ss} when compared to the native RecA. Such a comparison unraveled a component in RecA-Klenow interaction that is more specific to active RecA and is not shared by either an inactive RecA or any other arbitrary protein.

Further strong evidence for the specific interaction of RecA with Klenow fragment was provided by time-resolved fluorescence measurements. The kinetics of decay of fluorescence intensity of PySCl that is covalently linked to Klenow fragment showed a marked change in the presence

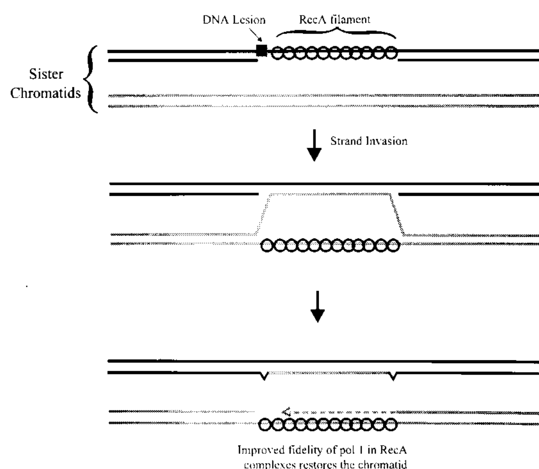


Figure 7. A Model Depicting Collaboration between RecA and Pol I during Postreplicational Repair
At the site of a postreplicational gap, the displaced strand gets coated by RecA following strand transfer (each circle is a RecA monomer). Pol I restores the gap in sister chromatid by high fidelity synthesis (dashed line).

of RecA (Figure 6B). The mean life time ($\tau_m = \Sigma\alpha_i\tau_i$) decreased from 4.67 ns to 3.77 ns in the presence of a 5.3 fold molar excess of RecA (Table 2). In contrast, the decay kinetics were unaffected by the addition of either lysozyme or BSA at the same molar excess (Figure 6B and Table 2). (A minimum of three exponentials were required for fitting the intensity decays in both the cases. This is not surprising when we realize that the fluorescence labeling might have occurred at more than one site and perhaps at several sites on the Klenow fragment and thus led to heterogeneity in fluorophore population. However, such a heterogeneity in fluorophore population is unlikely to affect our interpretations. The decrease in the value of fluorescence lifetime of PySCl from an expected single lifetime of 48 ns when it is free to 4.67 ns upon its covalent attachment to Klenow fragment could be due to its interaction with other side chains in Klenow leading to quenching of fluorescence). It is to be noted that r_{ss} is a function of both the fluorescence lifetime (τ) as well as the rotational correlation time (ϕ) as given by Perrin's equation,

$$r_{ss} = r_o /(1+ \tau/\phi)$$

where r_o is the maximum anisotropy. Therefore the observed increase in r_{ss} by the presence of RecA could have come about due to changes in τ as well as ϕ. However calculations show that the observed decrease in fluorescence lifetime (τ) from 4.67 ns to 3.77 ns due to RecA (Table 2) cannot account fully for the observed concomitant increase in r_{ss}. Therefore the increase in anisotropy arises mostly from an increase in the value of ϕ caused by RecA binding to Klenow. Despite the strong indication of a direct interaction between Klenow and RecA, the stoichiometry of the interaction could not be established from the present experiments. Presumably, this was because of the self-aggregating nature of the RecA protein even in its native state led to an increase in anisotropy as a function of increasing concentration. Further studies on direct estimation of ϕ using fluorescent probes having longer lifetimes could give information on the apparent stoichiometry of binding.

Discussion

These experiments suggest that primer/template DNA that is bound by RecA serves as a better substrate than naked DNA for incorporation by Pol I. Such a comparison of Pol I fidelity was done by directly sequencing the products by the Maxam-Gilbert sequencing method. Appropriate controls demonstrated that RecA-treated DNA samples perfectly retain Maxam-Gilbert specificity of base-modifications and no spurious cleavages were seen (Figure 3). This method, although not a highly quantitative one, is more direct than cloning and sequencing as the replicated DNA is not passaged through a cell where mismatches may be lost to some extent due to repair. RecA and Klenow have been studied extensively for their dynamics of association with DNA (Kornberg and Baker, 1992; Kowalczykowski and Eggleston, 1994). RecA binds ss-DNA cooperatively to form a righthanded nucleoprotein filament. In fidelity experiments, the concentration of RecA (3 µM) chosen was sufficient to cover the template strand (10 µM) fully (Leahy and Radding, 1986). Moreover, the concentration of Klenow (0.12 µM) used in these experiments is also high enough to cover all 3' primer termini (0.09 µM). Hence, with a plausible setting where Klenow-bound primer-terminus is housed in the wide groove of RecA filament, one can visualise two distinct ways through which RecA and Klenow collaborate with each other. RecA might directly interact with Klenow to modulate its function. Alternatively, the RecA induced unwound conformation of DNA might function as a better substrate for proofreading by Klenow (Leahy and Radding, 1986; Egelmen, 1993). The former possibility was verified by testing whether RecA and Klenow were capable of directly interacting with each other even in the absence of DNA. Time-resolved and steady-state fluorescence measurement revealed that the decay of the total fluorescence intensity and the steady state fluorescence anisotropy of PySCl labeled Klenow were changed upon addition of RecA protein. Increase in fluorescence anisotropy had a specific

Table 2.

Sr.No	Sample	Steady state anisotropy	Fluorescence lifetimes[a] (ns)			Amplitudes			Mean lifetime[b] (ns)	
		r_{ss}	τ_1	τ_2	τ_3	α_1	α_2	α_3	τ_m	χ^2
1	Klenow-pyr (1.5µM)	0.216±0.007	8.80	1.75	0.27	0.45	0.34	0.21	4.67	1.15
2	Klenow-pyr:RecA (1:5:3)	0.295±0.006	8.50	1.69	0.33	0.37	0.28	0.34	3.77	1.12
3	Klenow-pyr:lysozyme (1:5:3)	0.244±0.006	8.76	1.87	0.28	0.43	0.31	0.25	4.45	1.07
4	Klenow-pyr-BSA (1:5:3)	0.256±0.007	8.30	1.96	0.30	0.49	0.26	0.25	4.67	1.09

[a]The errors associated with τ_i and α_i are <10%
[b]The error associated with τ_m is <5%

component stemming from the active form of RecA as well as a non-specific component (Figure 6A). RecA that was irreversibly inactivated by extensive crosslinking showed a non-specific component in the increase of anisotropy that was essentially similar to that shown by non-specific binders. In the past, others have also noticed similar specific and non-specific components in binding through studies employing changes in fluorescence anisotropy. For instance, in analyses involving the binding of *E. coli* RNA-polymerase with cyclic AMP receptor protein (CRP), about 1/3rd to 1/2 of the anisotropy increase was non-specific and the remaining biologically relevant as exemplified by mutant and wild type CRP comparisons (Heyduk *et al*., 1997). Measurement with a picosecond laser and a time-correlated single photon counting system revealed that the average lifetime of fluorescence of Klenow-Pyr dropped from 4.7 ns to 3.8 ns specifically with the addition of native RecA. Under the same conditions, nonspecific binders had no effect on average lifetime of the fluorophore (Figure 6B, Table 2). These results strongly suggest the capability of RecA to directly and specifically interact with Klenow even in the absence of DNA. These observations open up the possibility that RecA might indeed be modulating Klenow fidelity by a direct protein-protein interaction.

Various *in vitro* studies have suggested that Klenow (or any polymerase for that matter) achieves its overall fidelity by distinguishing between the addition of a wrong dNTP and the right one at multiple steps of the reaction pathway (Echols and Goodman, 1991). On the other hand, RecA-filaments by themselves are poor editors of base pairing because the RecA pairing reaction tolerates a fair amount of DNA mispairing (Karthikeyan *et al*., 1998). This opens up a quandary of how RecA-filament improves Klenow fidelity despite its promiscuity in pairing DNA bases. A plausible solution to this can be reasoned as follows: Direct interaction between RecA and Klenow might affect Klenow in such a way that its exonucleolytic function is improved which is revealed by its enhanced proofreading in stalled complexes formed in RecA-filaments versus those that are devoid of RecA (Figure 5). This reasoning is also consistent with the finding that when RecA reduces the fidelity of translesion synthesis of Pol III in another biological setting (see below), it does so by inhibiting the 3' - 5' proofreading function of the epsilon subunit of DNA Pol III (Lu *et al*., 1986). Fidelity improvement by Klenow described here is specific to RecA-DNA complexes. The same DNA substrates when bound by *E. coli* SSB showed only a marginal improvement, if any, in fidelity compared to naked DNA substrates. These controls showing lack of any significant effects by *E. coli* SSB on Klenow mirror earlier observations where it was seen that while *E. coli* SSB markedly stimulates T7-polymerase, it fails to do the same for Klenow (Michaels *et al*., 1986). Also, as shown in Table 1, none of these conditions altered the rate of synthesis of DNA by Klenow. Thus the enhanced fidelity was not due to a slower rate of synthesis.

During postreplicational DNA repair, gaps opposite damaged regions are filled by RecA-mediated strand exchange from the sister-homologue (Rupp *et al*., 1971) failing which chromosomes degrade (Skarstad and Boye, 1993). Consequently, an equivalent gap is generated on the sister itself which needs to be precisely restored by Pol I synthesis. The RecA nucleoprotein filament that brings about strand exchange might play a critical role in enhancing the fidelity of Pol I synthesis at this step (Figure 7). Precise restoration of sister-strand by Pol I becomes a very strong necessity to ensure faithful propagation of chromosomal information. It is here that RecA filament positively modulates Pol I in such a way that the error-rate of Pol I is reduced as suggested by the *in vitro* results described here.

What are the implications of the results described here on the lethal phenotype of *recA*,*polA* double mutant? Current molecular explanation for this phenotype is that when a replication fork approaches nicks or gaps in the DNA, the fork collapses (Kogoma, 1997). In *recA* and *polA* double mutants such collapses go unrestored and lead to lethality. Such a model presupposes no direct collaboration between RecA and Pol I. In fact, such double-strand break (DSB) repair pathways are both RecA dependent as well as independent (Kogoma, 1997). It is therefore conceivable that the situations where RecA and Pol I physically collaborate with each other presumably along with other proteins have more to do with postreplicational strand-exchange repairs than DSB-repairs.

Experimental Procedures

Materials

RecA and SSB proteins were purified as described (Shibata *et al*., 1981; Lohman *et al*., 1986). Klenow fragment was overexpressed and purified as described (Joyce and Derbyshire, 1995). T4-polynucleotide kinase was from Amersham Lifescience. Adenosine triphosphate, phosphocreatine, creatine phosphokinase, dithiothrietol and all Maxam-Gilbert sequencing chemicals were from Sigma. Pyrene sulphonylchloride (PySCl) was purchased from Molecular Probes.

DNA Substrates

Single-stranded oligonucleotides were synthesized, end-labeled, and purified as described (Rao *et al*., 1993). Standard reaction conditions and assays are as described in figure legends.

Crosslinking of RecA

Intermolecular crosslinking of RecA was done using glutaraldehyde as described (Harlow and Lane, 1998). The extent of crosslinking was monitored by SDS-PAGE, which showed the presence of very high molecular weight products that barely entered the gel and complete absence of RecA monomers indicating that essentially all RecA monomers were crosslinked extensively. Following crosslinking, leftover glutaraldehyde was removed by extensive dialysis against 20 mM Tris-HCl (pH 7.5) and the sample was spun at 12000g for 20 min to remove any aggregates present. There was no detectable precipitation of RecA during crosslinking because almost entire RecA was recovered in the supernatant following crosslinking as measured by protein estimation.

Labeling of Klenow

The conjugation of Klenow with PySCl (an amine-selective reagent) (Brinkley, 1992) was done by reacting of PySCl (200 µM in acetone) with Klenow (20 µM) at 4 °C for 1-2 hours in 50 mM sodium bicarbonate (pH 9.2) containing 10 mM $MgSO_4$ and 1 mM DTT. Unincorporated PySCl was removed by gel filtration on a Sephadex G25 column which was equilibrated with 20 mM TrisHCl (pH 7.5), 10 mM $MgSO_4$ and 1 mM DTT. Complete removal of unincorporated and noncovalently bound PySCl was checked by repeated passage of the reaction mixture through Sephadex G25 column. The ratio of fluorescence intensity to protein concentration remained constant after each passage through the column indicating complete removal of noncovalently bound PySCl. Protein concentration was measured using the Bradford assay and the extent of labeling was monitored using fluorescence intensity (excitation at 350nm and emission at 382nm). The emission maximum of the fluorophore shifted from 376 nm to 382 nm upon its coupling to the Klenow fragment. The specific activity of the modified protein (Klenow-Pyr) was the same as the unmodified protein as assayed in primer extension assays (data not shown).

Fluorescence Measurements

Steady-state fluorescence measurements were carried out by using a SPEX Fluorolog FL1T11 T-format spectrofluorometer. Fluorescence anisotropy

was measured by monitoring the emission at parallel and perpendicular polarizations simultaneously by the use of the T-format optical arrangement. This method enhances the precision of measurement by removing the problems associated with sample instabilities.

Time-resolved fluorescence decay of Klenow-Pyr was measured by employing a CW mode-locked frequency-doubled Nd-YAG laser-driven dye (pyridine-1) laser which generates 4-10 ps pulses (Periasamy *et al.*, 1988). The modified protein was excited by using the second harmonic output of an angle tuned KDP crystal at 357nm and the fluorescence decay was measured at 382 nm by using a time-correlated single photon counting setup coupled to a microchannel plate photomultiplier (Swaminathan *et al.*, 1996). All the measurements were carried out at 25 °C.

Analysis of Decay Curves

The fluorescence decay curves at the magic angle were analysed by deconvoluting the observed decay with the instrument response function at 357nm to the intensity decay function represented as a sum of three exponentials:

$I(t) = \Sigma\ \alpha_i \exp(-t/\tau_i)$, i=1-3 where I(t) is the fluorescence intensity at time t and α_i is the amplitude of the ith lifetime τ_i such that $\Sigma\alpha_i = 1$.

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