Real time fluorescence analysis of the RecA filament: implications of base pair fluidity in repeat realignment

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Abstract During recombination, when *Escherichia coli* RecA mediates annealing across DNA repeats, Watson–Crick chemistry can only specify the complementarity of pairing, but not the most optimal frame of alignment. We describe that although stochastic alignments across poly(dA) and poly(dT) can lead to sub-optimally annealed duplexes containing ssDNA gaps/overhangs, the same are realigned into an optimal frame by a putative motor activity of RecA [Sen et al. (2000) Biochemistry 39, 10196–10206]. In the present study, we analyze the nature of realignment intermediates in real time, by employing a fluorescent probe, 2-aminopurine (2AP), which can not only report the status of RecA on the unstacked duplex, but also the fluidity of bases in such a filament. Although known to display a lower affinity for duplex DNA, RecA seems to remain functionally associated with these sub-optimally aligned repeat duplexes, until the realignment approaches completion. Moreover, a comparison of 2AP fluorescence in repeat versus mixed sequences indicates that bases in a RecA repetitive DNA filament exhibit higher degrees of freedom that might mediate a 'non-planar hydrogen bonding cross talk' across the bases on either strand. We discuss a model to explain the mechanistic basis of realignment and its implications in signaling the end of homology maximization, which triggers RecA fall off.

Key words: RecA; DNA repeat; Realignment; 2-Aminopurine; Cross talk

1. Introduction

Escherichia coli RecA has been widely studied as a universal prototype of pairing enzymes [1–3]. RecA is important not only as a housekeeping gene but also as a genome surveillance protein when posed with DNA damages, since it plays an active role in recombination and repair [4,5]. The importance of such functions is underscored by a conservation of these activities across many eukaryotic counterparts of RecA [3,6]. In fact, the nucleoprotein filaments formed with RAD51–DNA complexes are structurally very similar to RecA–DNA complexes [3].

By virtue of their relative abundance in all genomes [7,8], pairing is likely to encounter large stretches of repetitive DNA. It is widely accepted that Watson–Crick complementarity dictates pairing efficiencies across two DNA strands. However, when pairing ensues across repetitive regions, the complementarity principle cannot specify the most optimal frame of alignment across the paired strands, and would eventually lead to frame misalignments. An optimal frame is defined as the one that aligns repeats such that the adjoining mixed sequences automatically fall into a homologous register. One can envisage that any alignment that misses this optimal frame might lead to an abrogation of branch migration at the junctions of repeats and mixed sequences, due to a heterologous register [9]. In fact, prior studies have demonstrated that strand exchange slows down markedly when it encounters microsatellite repeats and this effect was directly proportional to the length of the repeat [10].

An extended DNA helix is the hallmark of a RecA–DNA filament, the structure of which seems to be highly conserved across evolutionarily distant species [11]. However, mechanistic implications of such a molecular model are still unclear. The fundamental signature of this RecA–DNA helix seems to be an enhanced rotational fluidity of DNA bases due to extensive base unstacking, which might play a role in homology recognition [12]. Here we describe another important implication of such a mechanistic facet. We had demonstrated earlier, that although pairing across repeats leads to misaligned duplexes, RecA can mediate a realignment of such mispaired frames to a maximally aligned register through a process that requires the energy of ATP hydrolysis [9]. In the present study, using 2-aminopurine, a fluorescent base analogue, as a reporter of RecA density in the filament, we demonstrate that in contrast to mixed sequences RecA dissociation from the realigning is much slower and depends on the status of frame alignment. In addition, RecA association on a poly(dA):(dT) repeat duplex seems to impart an element of fluidity to the paired bases which is reported by an enhanced yield of steady state fluorescence of the paired base. A model involving 'non-planar hydrogen bonding cross talk' across the paired strands has been described to explain the novel repeat realignment activity of RecA.

2. Materials and methods

2.1. Materials

RecA protein was purified as described [13]. T4 polynucleotide kinase and T4 DNA ligase were purchased from Amersham Pharmacia

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Abbreviations: ATP, adenosine triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); 2AP, 2-aminopurine; BSA, bovine serum albumin; dsDNA, double stranded DNA; DNase I, deoxyribonuclease I; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SSB, single stranded DNA binding protein; ssDNA, single stranded DNA

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Biotech. Adenosine triphosphate, phosphocreatine, creatine phosphokinase, dithiothreitol and nuclease-free BSA were purchased from Sigma. ATPγS was from Boehringer Mannheim.

2.2. DNA substrates

Single stranded oligonucleotides were purchased either from The Keck Biotechnology Resource Laboratory (Yale, USA) or from DNA Technology (Denmark). All oligonucleotides were purified on denaturing polyacrylamide gels and desalted further as described [14]. The purity of an oligonucleotide was judged by ^{32}P labeling the 5′ end by T4 polynucleotide kinase, followed by analysis of a 10% denaturing polyacrylamide gel which revealed that they were more than 90% pure. DNA was quantified by measuring the absorbance at 260 nm and expressed as total nucleotide concentration. The oligonucleotides used are as listed in Table 1.

2.3. End labeling of oligonucleotides

Oligonucleotides were end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP as described [14].

2.4. Reaction conditions

2.4.1. Gel assays. Reactions involving radiolabeled substrates were performed using A_{30} tester (3.4 μM) and T template (8 μM) and tether (2.7 μM) (cold kinased). Pre-synapsis was done by incubating the template with RecA (2.8 μM) at 37°C for 10 min in an ATP regeneration buffer (33 mM Tris–HCl (pH 7.5), 1.2 mM magnesium acetate, 2 mM DTT, 1.2 mM ATP, 8 mM phosphocreatine, creatine phosphokinase (10 U/ml) and nuclease-free BSA (100 μg/ml)) followed by annealing with tester and tether. Thermally annealed duplexes were obtained by denaturing the oligonucleotides at 85°C for 2 min in 10 mM Tris–HCl (pH 7.5) and 10 mM magnesium acetate, followed by cooling to room temperature (~25°C) over a period of 45 min. Subsequently the composition of thermally annealed samples was adjusted to that of ATP regeneration buffer. Gaps were scored by the addition of several-fold molar excess of labeled competitor, as specified. For both thermal and RecA reactions, ligation was initiated by the addition of T4 DNA ligase (300 U/ml) and magnesium acetate (10 mM). Aliquots were withdrawn at various time points and quenched by the addition of an equal volume of loading buffer (20 mM EDTA, 0.5% SDS and 95% formamide) with appropriate indicator dyes. The samples were analyzed on a 10% denaturing polyacrylamide gel electrophoresed at 50°C.

2.4.2. Fluorescence assays. RecA pre-synapsis was done as described above. The pairing reactions were initiated in an ATP regeneration buffer at 25°C, by the addition of either naked (described as thermal) or RecA coated template (12 μM template, 4 μM RecA) to the 2AP tester (5 μM). Subsequently the reactions were monitored over a time course for changes in fluorescence intensities. Such a time course analysis also involved steps where non-fluorescent homologous competitor (tester, 40 μM) and template DNA (120 μM) were added at specified stages of the reaction (see legends).

2.5. Fluorescence measurements

The fluorescence measurements were carried out by using a SPEX Fluorolog FL1T11 spectrofluorometer. 2AP fluorescence was monitored at 25°C in 150 μl reactions (in ATP regeneration buffer) by exciting at 310 nm and measuring the emission at 370 nm. All fluorescence intensities were corrected for variable background emissions and lamp fluctuations (signal/reference: *s/r*). Hence, all fluorescence intensities are plotted as *s/r* values.

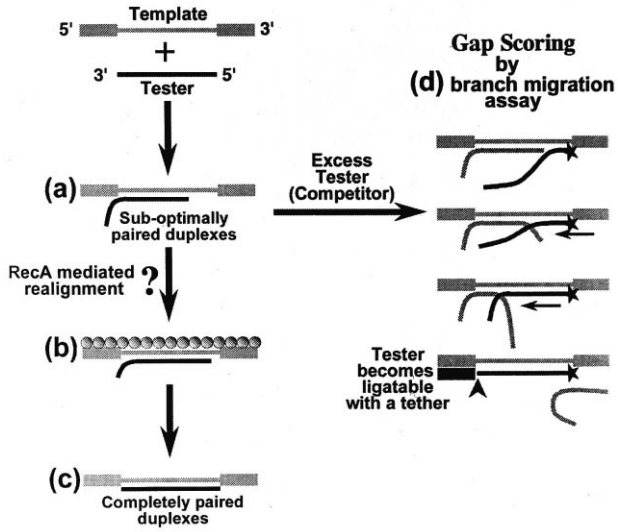


Fig. 1. A schematic representation of the experimental design for scoring gaps in misaligned duplexes. The gray and the black lines depict the template and tester strands respectively. Both thermal and RecA mediated pairing across repeat sequences (thin lines) may result in sub-optimally paired duplexes with gaps (a). The gaps were scored by adding molar excess of labeled homologous tester as competitor; the excess of homologous tester added anneals at the gap and juxtaposes to form a migratable branch that eventually becomes ligatable with a pre-annealed mixed sequence tether (thick line, branch migration assay (d)). The arrowhead indicates a ligatable nick. Although stages (a), (b) and (c) are parts of RecA mediated realignments, RecA filament (gray circles) is shown only in (b). How does RecA filament sense the presence of 'short' gaps in such sub-optimally aligned duplexes and modulate the status of RecA density vis a vis that of realignment ((b) to (c))?

3. Results

Across repetitive DNA tracts, the chemistry of complementary annealing cannot specify an optimal frame of alignment because of which pairing is likely to be a stochastic event. However, the relative abundance of repeats in genomes demands that there exists a machinery by which the annealed frames can be optimized to a maximal homology register. Can *E. coli* RecA, a universal paradigm for pairing enzymes, bring about such 'maximization'? We tested this in a simple experimental system involving complementary pairing between poly(dA)/poly(dT) repeat strands. The basic experimental design is as depicted in Fig. 1. Using the same experimental system, we had shown earlier by targeted ligation assay (TL assay) that RecA mediated annealing between DNA repeats shows a slow realignment of frames following an initial stochastic mode of pairing [9,14]. This was revealed by a time dependent

Table 1

Oligonucleotide	Sequence (5′ to 3′)
T template	acgcacatactaggctgtat**ttttttttttttttttttttttttttttttt**ttcagtacagtcatgacagt
A_{30} tester	aaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
2AP-A_{30} tester (i)	aaaaaaaaaaaaaaaaaaaaaaaaa(2AP)aaaa
2AP-A_{30} tester (ii)	aaaaaaaaaaaaaa(2AP)aaaaaaaaaaaaaaa
2AP-A_{30} tester (iii)	aaaa(2AP)aaaaaaaaaaaaaaaaaaaaaaaaa
Tether	atacagcctagtatgtgcgttag
Mixed seq. template	ttgataagaggtcatttttgcggat**ggcttagagcttaattgctgaatctggtgctgt**agctcaacatgttttaaatatgcaa
Mixed seq. tester	acagcaccagattcagcaattaagctctaagcc
2AP mixed seq. tester	acagcaccagattcagcaattaagctct(2AP)agcc

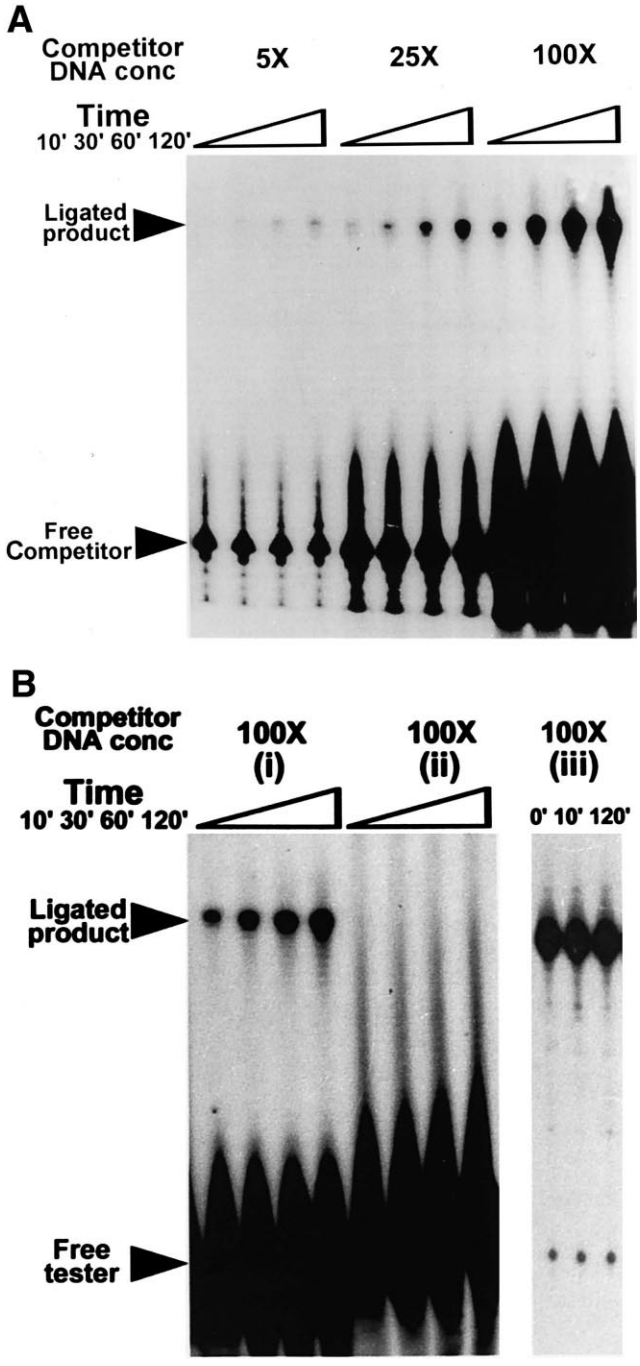


Fig. 2. Analyses of gaps in paired frames across repeats: a comparison of thermal pairing versus RecA mediated realignment: (A) Branch migration assay for thermally annealed duplexes: The experimental design is as depicted in Fig. 1 wherein the initial thermal pairing was done with T template, cold kinased tether and unkinased A_{30} tester (Section 2) followed by a challenge with increasing molar excess of labeled homologous tester in three independent reactions (5×, 25× and 100×). After incubation for 15 min, T4 DNA ligase was added (0 min) following which aliquots were withdrawn at specified time points and quenched with loading buffer. (B) RecA mediated realignment of sub-optimally paired duplexes to a maximized frame – analysis of branch migration assay. Two RecA mediated pairing reactions ((i) and (ii)) were monitored as a function of time using the branch migration assay as described in Fig. 1 and A. Pairing was carried out using unkinased A_{30} tester and cold kinased tether. 100-fold molar excess of ^{32}P labeled homologous competitor (A_{30} tester) was added either after 10 min (i) or 120 min (ii) of pairing. After 15 min of competition, T4 DNA ligase was added (0 min) following which aliquots were withdrawn at specified time points and quenched with loading buffer. Set (iii), a control reaction, involves initial pairing (for 120 min) using a labeled tester followed by competition with an unlabeled tester (100×). Aliquots, withdrawn at specified time points of competition, were treated with ligase for 10 min and quenched with loading buffer.
←

emergence of 'ligatable frames' specifically in RecA reactions, where the labeled tester is realigned towards a 'fixed frame' of pre-annealed tether. Thermally annealed strands showed no such time dependent changes. Therefore the study essentially monitored the progression of realignment from stage a to c, as described in Fig. 1. A simple inference from such a study was that, in contrast to that of RecA reaction, the residual ssDNA gaps remained unrectified in thermally annealed duplexes (a in Fig. 1) due to lack of frame realignment. Is there an active correlation of RecA density on the realigning intermediates vis a vis the abundance of the gaps being rectified? Can RecA sense the presence of ssDNA gaps/overhangs and persist onto such sub-optimally aligned duplexes to mediate complete realignment? In this study, we designed a gap scoring assay that directly tests the presence/absence of such putative ssDNA gaps during realignment. Furthermore, the study also elaborates on a plausible mechanistic relationship between realignment vis a vis the status of RecA in these complexes, by employing a real time fluorescence assay.

3.1. Analysis of sub-optimally paired DNA repeats: gap scoring by branch migration assay

The basic premise of the gap scoring assay is that gaps in the repeat regions (a in Fig. 1), if any, can be captured by adding several-fold molar excess of homologous competitor strand (A_{30} tester), simply by law of mass action (d in Fig. 1). It must be noted that, preceding such a competition, the initial pairing between T template and the unlabeled A_{30} tester was carried out at equimolar ratios, conditions under which annealing in both thermal as well as RecA reactions went to completion within 5 min as monitored by parallel native gel assays performed with ^{32}P labeled testers ([9], equivalent data in Fig. 3A). Thus the competition experiments, described below, were performed on preformed duplexes that are likely to contain gaps due to frame misalignments.

Upon challenge, the molar excess of labeled competitor lands on an existing gap to form a branch migratable complex with the pre-annealed tester. Following branch migration, the labeled competitor (A_{30} tester) aligns into a ligatable frame with a tether (d in Fig. 1). Using such an assay, we studied the status of gaps in thermally annealed duplexes between T template and A_{30} tester (in the absence of RecA) and compared the same in RecA mediated duplexes. Thermally annealed duplexes were challenged by varying doses of labeled competitor for 15 min following which ligase was added to score the competed products. This assay follows a time course of ligation that captures the cumulative signal of branch migration products in the population and thereby reveals an increase over time (Fig. 2A). In addition, as expected, the mass action of competitor reveals as a dose dependent increase of ligation products. Both these trends are consistent with the process of

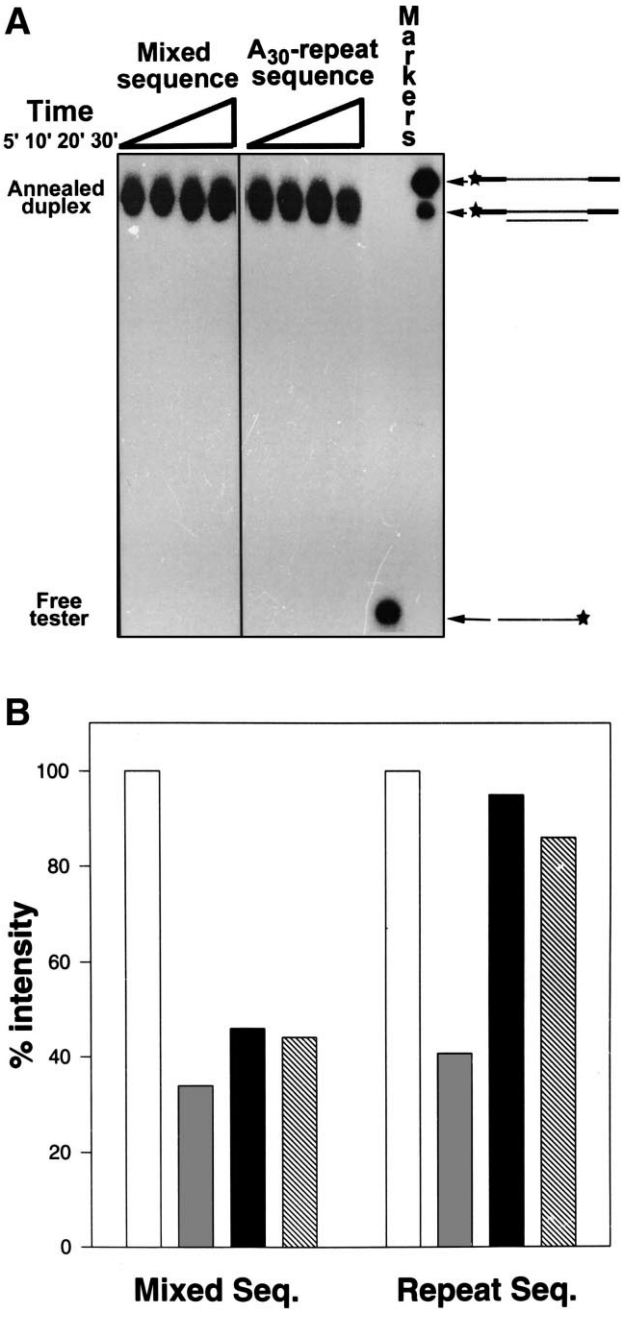


Fig. 3. A: Analyses of annealing efficiencies by native gel electrophoresis: Two independent RecA pairing reactions were performed (Section 2), one with mixed sequence substrates (2AP mixed seq. tester with mixed seq. template, Table 1) and the other with repeats (2AP-A_{30} tester (i) with T template, Table 1). Both reactions were initiated using 5′-^{32}P labeled testers, following which aliquots were withdrawn at specified time points and deproteinized by proteinase K (100 μg/ml) in 0.5% SDS, 20 mM EDTA (at 37°C, 20 min) before analysis on a 10% native polyacrylamide gel. Adjoining marker lanes depict the positions of repeat DNA substrates and the annealed product, where the asterisk indicates the position of the ^{32}P label. B: A comparison of stacking interactions of (dA):(dT) pairs in repeat versus mixed sequences. A comparative analysis of fluorescence intensity (*s*/*r*) of 2AP mixed seq. tester versus 2AP-A_{30} repeat (ii) was carried out using ssDNA (white), naked dsDNA (gray) and RecA mediated duplexes (black). Parallel reactions, where thermally annealed duplexes (those represented by gray bars) were subsequently coated with RecA (in 1 mM ATPγS), were also analyzed (striped bar). Templates were coated with RecA in the presence of 1 mM ATPγS (under typical pre-synapsis reaction conditions, Section 2) and paired with respective 2AP testers for 15 min to generate stable RecA coated duplexes. Steady state fluorescence was monitored and plotted with respect to 2AP–ssDNA fluorescence. →

branch migration. One can note that the size of the ligated product (tether plus tester) was consistent with the process of branch migration [9].

Since at the highest concentration of competitor, i.e. 100×, the increase in ligation product as a function of time was most obvious, we analyzed the status of gaps in RecA mediated reactions under this condition. More importantly, we studied whether such gaps that may exist in stochastically formed early pairing intermediates, are repaired at a later time point, by a realignment process mediated by RecA, as demonstrated by us earlier [9]. Based on our own study [9] as well as others' [15,16], it is clear that a 100-fold molar excess of ssDNA competitor (tester) added can easily titrate RecA away from the paired complexes, thereby 'freezing' them during realignment. Paired complexes that retain gaps were subsequently scored by the branch migration assay. We compared the status of gaps at two different time points of the RecA reaction. RecA intermediates formed after 10 min of pairing revealed a considerable population of gapped duplexes as revealed by the appearance of a ligation signal (Fig. 2B(i)). In contrast, when such complexes were analyzed after 2 h of pairing (and realignment), no gaps were detected (Fig. 2B(ii)). Moreover, RecA mediated realignment was complete in the entire population as evidenced by absence of a ligation signal even after longest exposure to the competitor (Fig. 2B(ii), 120 min). One must note that failure to detect ligation products in this assay was not due to the depletion of ATP in the reaction, which could simply prevent ligase action thereby reporting a negative result. This was demonstrated in a parallel control where an identical reaction (as in Fig. 2B(ii)) was carried out with the following changes incorporated. (1) The initial pairing was carried out with a labeled tester that was followed by competition with unlabeled 100× tester. (2) Ligase efficiency was monitored at specific time points of competition by employing a fixed time of ligation as described below. Following a RecA mediated pairing reaction of 120 min, the reaction was challenged with a 100× dose of unlabeled tester (Fig. 2B(iii), 0 min). The ligation efficiency was measured by the addition of ligase to aliquots withdrawn from different time points of competition and subjecting them to a fixed time of ligation (10 min). This control essentially measures the efficacy of ligase at different time points of competition, following the long period (120 min) of RecA mediated pairing (and realignment) reaction. The ligation efficiency remained unchanged from start (0 min) to finish (120 min) of competition and hence ruled out the possibility of ATP depletion during the assay (Fig. 2B(iii)). Therefore, the results described in Fig. 2B(i) and (ii) reflect genuine differences in the status of gaps in early versus late time points of RecA realignment reactions. Previous experience with this oligonucleotide based RecA pairing system had indicated that, in spite of such long hours of incubation, due to relatively poor association of RecA with such short duplex substrates, ATP depletion is slow and hence can be successfully sustained by the ATP regeneration system employed. In fact, the control, just described, vouches for the

same. As described earlier [9], as well as in the preceding experiment (Fig. 2B(ii)), RecA mediated frame realignment is essentially complete by 2 h leaving no residual single stranded gaps in the repeats. We corroborate the same by an independent real time assay below and study the relationship between DNA strand dynamics and RecA status in the realigning complexes.

3.2. Fluorescence analysis: realignment versus the status of RecA filament

We were intrigued by the fact that, given the dynamic nature of the ATP hydrolyzing RecA filament on the short substrates used, RecA was still functionally associated with the misaligned duplexes, bringing about an active realignment. Our earlier DNase I protection experiments hinted to this effect where RecA–DNA complexes that were populated with incompletely realigned frames showed better protection of the T template strand by RecA than the corresponding complete duplex [9]. However, the temporal relationship between RecA in the filament and a repeat realignment was unclear since DNase I probing is more of a snapshot analysis. Therefore, we wanted to use a DNA based probe that is sensitive both to strand annealing as well as base stacking interactions in order to monitor RecA status vis a vis strand realignment, in real time. We thought that 2AP, an adenine isomer, whose fluorescence intensity is highly quenched within duplex DNA largely due to intrastrand base stacking interactions [17], serves this purpose. In fact, in addition to unstacking, 2AP fluorescence is also sensitive to the ability of the bases to rotate and thereby flip in and out of the helix, indicating higher degrees of freedom of the paired base [18,19]. Therefore, 2AP substituted oligonucleotides were used as testers (2AP-A_{30} tester) to pair with T template in the experiments described below. We repeated native gel analysis with these 2AP testers and confirmed that, as observed earlier, pairing in thermal as well as RecA reactions went to completion within about 5 min (data shown only for RecA, Fig. 3A). In fact, these testers behaved exactly similar to their non-fluorescent counterparts, as monitored by the branch migration assay (data not shown).

3.3. RecA mediated base unstacking leads to enhanced fluorescence emission of 2AP in repeat duplexes

To use 2AP fluorescence as a real time probe for monitoring RecA status on the filament, we tested whether the stable binding of RecA, in the presence of ATPγS, leads to an enhancement in fluorescence emission due to base unstacking. More importantly, we wanted to compare the nature of the 2AP base in a paired RecA–DNA complex for a mixed sequence versus that in a (dA):(dT) repeat stretch. We predicted that such a comparison might give us an insight about the differences in fluid nature of the 2AP base in such unstacked RecA–DNA helices. It was observed that, following thermal pairing, 2AP fluorescence in ssDNA was quenched to similar levels in both mixed as well as repeat sequences (2AP mixed seq. tester and 2AP-A_{30} tester (i), Fig. 3B). On the other hand, a similar comparison between duplexes formed by RecA mediated pairing was strikingly different. In RecA reactions, quenching was observed specifically in the mixed sequence duplex but not in the repeat duplex. In the latter, fluorescence emission was significantly higher than that of its thermal counterpart ($\sim$90%). It must be noted that RecA binding relieved fluorescence quenching to almost single stranded levels only in the repeat duplex (Fig. 3B). However, the enhancement observed for RecA mediated mixed sequence pairing was only marginal (10–15%). We measured fluorescence lifetimes of 2AP in our experimental system by the method described earlier involving picosecond laser pulse fluorimetry [20]. The observed multi-exponential nature of fluorescence decay of 2AP in DNA duplexes is similar to that reported [21]. Changes in the mean fluorescence lifetimes in RecA–DNA complexes (calculated from the fluorescence decay parameters, unpublished observations) are consistent with the observed changes in the steady state fluorescence intensity (Fig. 3B). Such a correspondence attests the reliability of using 2AP fluorescence intensity changes in the present study. Moreover, since RecA remains stably bound to DNA in ATPγS, the enhanced fluorescence observed in RecA reactions remained stable over several minutes as monitored by a time course (data not shown). The increase in fluorescence observed specifically in RecA repeat duplexes may stem from an enhanced fluidity of the unstacked bases in the repeat filament, assisted by an intrinsically higher propeller twist associated with such homopolymeric duplexes [22]. This demonstrated that 2AP fluorescence is not only a sensitive indicator of base pair unstacking but could be simultaneously used to monitor RecA density in paired complexes. In addition, 2AP–ssDNA fluorescence was not enhanced any further from its single stranded emission when it was coated by RecA in ATPγS (data not shown). However, thermally annealed duplexes that were subsequently coated with RecA (in ATPγS), again showed an enhancement in fluorescence reiterating the observation for RecA mediated pairing reactions above (Fig. 3B).

We also studied 2AP fluorescence in repeat duplexes formed by RecA mediated pairing in ATP hydrolyzing conditions. A spectral scan revealed that fluorescence emission from RecA paired duplexes was measurably higher than that from naked repeat duplexes without any significant shift in emission spectra (data not shown). However, it must be noted that the enhancement was not as high as in ATPγS, because under ATP hydrolyzing conditions RecA filament is more dynamic and hence the protein tends to fall off in homologously paired regions [9,23]. Nevertheless, we believe that the enhancement is sufficient for monitoring RecA status in the filament (in ATP hydrolyzing conditions) and can be used to study the same as a function of homologous pairing and frame realignments. In fact, the experiments described below bear out these suppositions.

3.4. RecA status in the filament: a time course analysis of pairing across mixed sequences

It is well established that RecA mediated complementary pairing across mixed sequences goes to completion very fast (Fig. 3A, [9,24]). Different approaches have demonstrated earlier that RecA, under ATP hydrolyzing conditions, has significantly lower affinity for duplex DNA [25] and probably falls off following homologous pairing and strand exchange [23]. We used the mixed sequence pairing reaction as a control to contrast the nature of these paired complexes versus those in repetitive sequences (that may contain gaps, Fig. 2). Given the uniqueness of the pairing frame involved in a mixed sequence reaction, gaps are unlikely to exist. As a prelude to studying repeat sequence realignments using 2AP fluores-

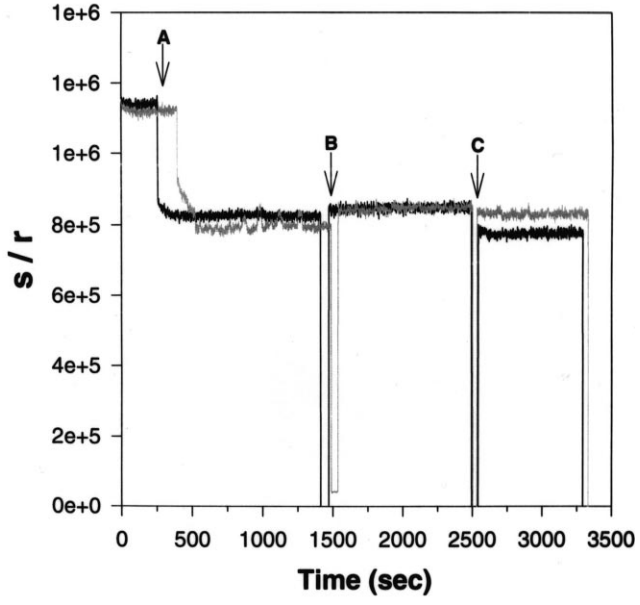


Fig. 4. A time course analysis of thermal and RecA mediated pairing across mixed sequences. 2AP mixed sequence tester (Section 2, Table 1) was annealed to the mixed sequence template under standard reaction conditions during which fluorescence intensity (*s*/*r*) was monitored as a function of time. Pairing was initiated by the addition of either naked template (black line) or RecA coated template (gray line) to a reaction containing 2AP mixed seq. tester ssDNA (step A). Paired complexes were challenged with excess of non-fluorescent mixed seq. tester (step B) followed by an addition of several-fold molar excess of the mixed sequence template (step C).

cence, we tested the experimental protocol using mixed sequence DNA as a control.

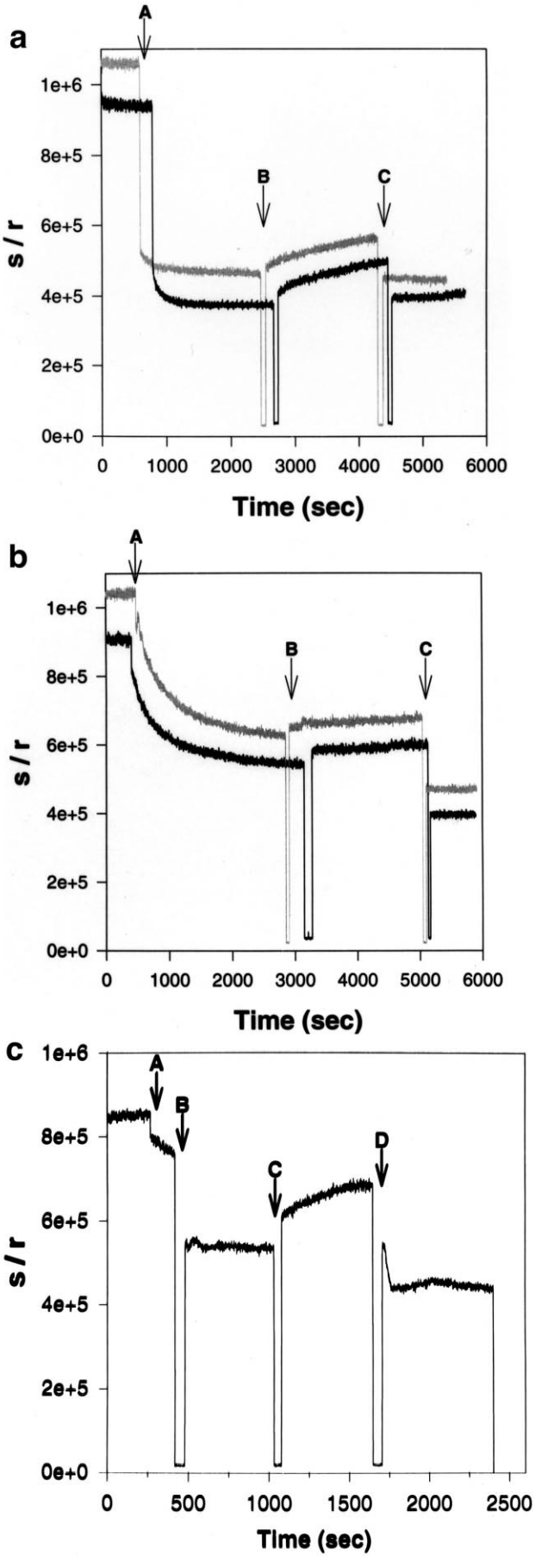
The designed protocol involves three steps of analysis: step A that initiates pairing; step B that tests the presence of gaps by 'chase' with a competitor, as a result of branch migration; and step C that monitors the end point of the reaction (w.r.t. sequestering of the unpaired 2AP testers as well as complete dissociation of RecA). The 2AP mixing seq. tester was paired with either a naked or RecA coated mixed seq. template (step A) and monitored over a time course. One must note that under similar conditions, as demonstrated in Fig. 3A, pairing was complete within 5 min (300 s). Both thermal and RecA reactions showed sharp decline in fluorescence intensities and achieved similar steady state levels, suggesting the likely dissociation of RecA concomitant with strand annealing (Fig. 4). When challenged with a non-fluorescent mixed sequence tester (homologous competitor without 2AP), both RecA as well as thermally annealed complexes were refractory to competition as was evident from the lack of fluorescence enhancement (Fig. 4, step B). An increase in fluorescence could have resulted only from a chase of the annealed 2AP tester by the added non-fluorescent competitor (as in Figs. 1(d), and 2A). Moreover, following competition, when several-fold molar excess of mixed sequence template was added, no further fluorescence changes were observed (Fig. 4, step C). Here step C was intended to monitor the end point of the reaction which would manifest as a decrease in the fluorescence due to the following likelihoods: (a) residual RecA on annealed duplexes, if any, will be titrated away by molar excess of ssDNA competitor added [15,16], (b) pairing of either free or released 2AP testers (from step B). The lack of change in fluorescence suggested that most of the paired 2AP testers were probably denuded of RecA (by step C). These results taken together therefore indicate that, as expected, pairing across mixed sequences is fast and leaves no gaps in the annealed duplexes. Moreover, concomitant with fast pairing (thermal), RecA fall off from such duplexes also seems to follow similar fast kinetics. The above results corroborate the findings described earlier where TL assay showed no gaps in mixed sequence pairings in both thermal as well as RecA reaction [9].

3.5. Real time analyses of RecA status during realignment of repeats

Pairing across repeat sequences can be visualized as a two-step process: (a) an initial stochastic mode of homologous pairing and (b) a maximization of paired frames by realignment. These two steps are virtually inseparable when one considers a mixed sequence pairing because of the unique frame of alignment. Hence, in the mixed sequence control described above, it was not possible to address the role of these steps in triggering RecA dissociation from the paired complexes. To address this issue, we repeated 2AP fluorescence analysis on repeat duplexes. Moreover, to establish the conditions where 1:1 complexes of repeat duplexes (T template:2AP-A_{30} tester) are formed, we titrated a fixed concentration of the tester with increasing concentrations of template. RecA and thermal pairings were carried out in parallel. Pairing was monitored by the decline in fluorescence intensity as a function of template/tester ratio. In both reactions, pairing saturated at about 1:1 ratio (data not shown), suggesting that both thermal as well as RecA reactions yielded similar complexes.

In addition to the experimental protocol described above (as in Fig. 4), we analyzed the position effect of 2AP, if any, in pairing versus realignment. Three separate 2AP testers were designed: one where the 2AP was positioned in the middle (2AP-A_{30} tester (i)) and the other two, five nucleotides away from either the 3′ or 5′ end (2AP-A_{30} testers (ii) and (iii) respectively). At step A, where pairing was initiated, thermal reactions showed a steep decline in fluorescence intensity (Fig. 5a) which was in sharp contrast to the slow and steady decrease observed for the RecA reactions (Fig. 5b). This slow decline can only stem from a slow dissociation of RecA from the realignment intermediates, and hence is a direct measure of the status of RecA during realignment. In addition, all the three 2AP-A_{30} testers that differed in the location of 2AP showed exactly similar kinetics in thermal as well as RecA reactions (data shown only for 2AP at 5′ and middle location to avoid crowding). There was no effect of 2AP position on the fluorescence pattern of the reactions following either step A or the subsequent steps (see below). The steep decline followed by no further time dependent changes in fluorescence, observed for thermal pairing across repeats (Fig. 5a), was similar to that observed for mixed sequence pairing (thermal and RecA, Fig. 4). Moreover, during the time period of observation, the extent of fluorescence quenching was less in RecA reactions compared to that of thermal (Fig. 5b versus a). However, the differential kinetics observed in RecA versus thermal reactions does not reflect differences in the pairing rates. This was demonstrated earlier by a native gel assay, where pairing between both repeat as well as mixed sequence substrates was complete by the earliest time point analyzed (5 min, Fig. 3A). These observations suggest that the steady

Fig. 5. A time course analysis of pairing between 2AP-A_{30} tester and T template: thermal (a), RecA (b). The experimental protocol is identical to that described in Fig. 4 with the following changes. In two separate reactions, T template was paired with 2AP-A_{30} testers differing in the location of the 2AP probe, one at the 3′ end (2AP-A_{30} tester (i), gray line) and the other at the center of the ssDNA tester (2AP-A_{30} tester (ii), black line) (Section 2, Table 1). (Initiation of pairing (step A), challenge with excess non-fluorescent A_{30} tester (step B) and addition of several-fold molar excess of T template (step C)). c: The effect of RecA loss from sub-optimally paired complexes at an early time point during realignment. The reaction is identical to that described in Fig. 3A with 2AP-A_{30} tester (i). The pairing was initiated by a RecA coated T template (step A), followed by addition of EDTA (25 mM, step B). Subsequently, an excess of non-fluorescent A_{30} tester was added as competitor (step C) followed by several-fold molar excess of T template (step D). →



decline in fluorescence intensity observed in RecA reactions stems from a slow progressive loss of RecA from the paired filament. Therefore, we infer that RecA fall off is an imminent step that follows repeat realignment (see Section 4).

At step B, where the non-fluorescent homologous competitor was added, the outcome was different in thermal versus RecA reactions (Fig. 5a,b). As expected, the thermally annealed products were competable by the non-fluorescent tester indicated by the slow and steady increase in the fluorescence intensity. In contrast, the corresponding RecA counterparts were hardly competable. As described in Fig. 1 and demonstrated in Fig. 2A,B, the competability stems from resident gaps that initiate a branch migration leading to the displacement of the 2AP tester from the complex, thereby leading to a steady increase in fluorescence. These results therefore suggest that thermally annealed duplexes are sub-optimally paired and hence retain gaps, whereas the same in RecA paired complexes are rectified by realignment.

To assess whether RecA was still associated with the intermediates during realignment, we added several-fold molar excess of ssDNA T template as competitor that would not only titrate RecA away but also quench all free ssDNA testers, if any. At step C, fluorescence intensity in thermal reactions (due to free 2AP testers) reverted back to the paired state (as before step B). However, in contrast, RecA reactions revealed a larger decline in fluorescence, that stems from RecA dissociation rather than sequestering of the free 2AP testers. It must also be noted that the end point attained at step C was identical in both RecA as well as thermal reactions indicating the complete dissociation of RecA (Fig. 5a versus b).

The experiments described in Fig. 5a,b led us to believe that, in contrast to thermal pairing that retains gaps, RecA rectifies them by realignment which ultimately leads to a slow and progressive dissociation of RecA from the realigned products. This conclusion hinges on the following interpretations: (a) the slow decrease in fluorescence is due to RecA dissociation and (b) lack of competitor chase reflects no detectable gaps in the realigned products. We confirmed this interpretation in a control; firstly we tested whether a deliberate 'kick off' of RecA from the early intermediates of realignment leads to a steep fall in the fluorescence; secondly whether such RecA denuded realignment intermediates reveal the presence of gaps following a competitor chase. On both counts, the control experiment bore out our original interpretations as explained below. Upon dislodging RecA soon after initiating pairing (step B in Fig. 5c), the fluorescence intensity declined

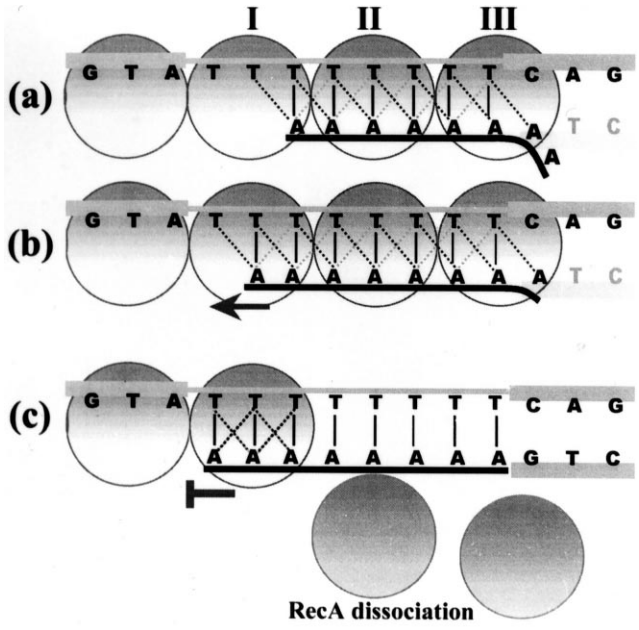


Fig. 6. A model depicting the 'hydrogen bonding cross talk' during RecA mediated realignment. The RecA coated top strand represents the template where a (dT) repeat is flanked by mixed sequences on either side. a: A (dA) tester pairs with (dT) repeat stochastically leading to unpaired (dT) and (dA) bases at monomers I and III respectively, in the RecA filament. The solid lines between the (dA) and (dT) residues represent complementary pairing mediated by Watson–Crick hydrogen bonds. The dotted lines across the paired strands depict a hydrogen bonding cross talk with the adjacent complementary bases. b: An intermediate of realignment where the (dA) tester has slid leftwards (indicated by the arrow) by a single base. c: Realignment places the (dA) tester in an optimal register juxtaposing it to the adjoining tether. Due to the presence of adjoining mixed sequences, immediate to such an optimal frame, further cross talk across the strands is unproductive, signaling the completion of realignment and concomitant dissociation of RecA.

steeply, indicating that the original fluorescence was almost entirely due to the presence of RecA. This observation underscores the property of RecA mediated unstacking of bases that relieves the fluorescence quenching in paired 2AP duplexes (Fig. 3B). Moreover, 2AP testers in such RecA denuded DNA intermediates were efficiently competable by a non-fluorescent competitor, a result highly reminiscent of thermally annealed products (compare step C, Fig. 5c with step B, Fig. 5a). Thus, when RecA reaction was prematurely abrogated, it mimicked thermally annealed sub-optimally aligned products, thereby indicating the active role of RecA in repeat realignments. Again, as expected, an addition of excess template scored the end point of the reaction, where the intensity dropped to the minimum levels (step D, Fig. 5c).

4. Discussion

Thermal pairing between T template and A_{30} tester leads to sub-optimally aligned duplexes as shown here by the branch migration assay (Fig. 2A). Such sub-optimally aligned duplexes are rectified in a realignment reaction catalyzed by RecA (Fig. 2B). In fact, previous studies indicated that this is an ATP hydrolysis dependent process and ensues across mono- di- as well as trinucleotide repeat stretches [9]. The focus of this study is to get a mechanistic insight on the relationship between realignment vis a vis the status of RecA in these complexes.

We used a sensitive probe that can report the density of RecA in the filament in real time. 2-Aminopurine (2AP), a fluorescent base analogue, is very sensitive to conformational changes in DNA structure [26,27]. 2AP fluorescence is highly quenched in polynucleotides and duplex DNA due to stacking interactions with the neighboring bases. However, if the DNA stacking around the 2AP base is perturbed, the fluorescence emission is recovered as a fold enhancement which depends on the amount of unstacking and increases dramatically if the base is flipped out of the DNA helix [19,28]. Moreover, background fluorescence from tyrosine and tryptophan residues in proteins does not interfere with 2AP fluorescence due to the non-overlapping nature of the excitation and emission spectra. Hence, we surmised that 2AP could serve as a good probe for monitoring RecA–DNA interactions where RecA binding to the 2AP duplex would lead to an enhancement of the fluorescence due to base unstacking. Here we demonstrate that, unlike that of mixed sequences, RecA association (in ATPγS) on a (dA):(dT) repeat duplex uncovers almost the entire fluorescence associated with 2AP-A_{30} tester, as an enhancement (Fig. 3B). Although extrahelical base flipping cannot be ruled out, the 2- to 3-fold enhancement observed here probably reflects on the unstacking and freedom of the bases within the RecA–dsDNA filament, since base flipping is known to cause a much higher increase in fluorescence intensity (31–54-fold, [19]). It is known from previous studies that 2AP fluorescence enhancement in protein–DNA complexes is associated with different states (stacking) of the base within the complex. In fact, it is envisaged that the base undergoes a series of transitions from a paired fully stacked to a paired partially stacked to an unstacked to a final unpaired form, the latter also being able to flip out of the helix [21,27]. A spectral scan revealed that in ATP hydrolyzing conditions, fluorescence emission associated with RecA paired duplexes was measurably higher than that from naked DNA duplexes. Furthermore, a comparison of mixed and repeat sequences revealed that bases seem to experience higher degrees of unstacked freedom in the RecA coated repeat duplex (Fig. 3B). Does this contrast in fluorescence enhancement point out to the enhanced fluidity of the 2AP base pair specifically within the repeat stretch, when it is in a RecA filament? Picosecond rotational dynamics of 2AP observed through time resolved fluorescence depolarization does indeed suggest that RecA binding results in an increased fluidity of the 2AP base pair (unpublished observations).

Stochastic pairing, in both thermal and RecA reactions, is rapid when measured across mixed as well as repetitive sequences. This was expected because the pairings being monitored here depend entirely on complementary annealing of bases which is likely to be as efficient between naked DNA strands as it is in RecA mediated reactions (Fig. 3A). 2AP fluorescence monitored in thermal versus RecA pairing reactions across mixed sequences showed very similar kinetics (Fig. 4). The rapid decline of fluorescence in RecA reactions suggested that the protein dissociates from the complexes soon after pairing. Moreover, this dissociation seems to be complete as no further decrease in fluorescence was observed following RecA chase by an excess ssDNA competitor (step C, Fig. 4). These results came in sharp contrast when we compared RecA status during realignment of repeat duplexes. Thermal annealing across the repeats, which resulted in a

rapid decline in fluorescence intensity, was similar to that observed for mixed sequences (compare step A in Fig. 4 versus Fig. 5a). This indicated that the pairing rates were similar across both mixed as well as repeat sequences. However, the kinetics of fluorescence decline associated with RecA–repeat DNA complexes was rather slow (Fig. 5b) and not commensurate with rapid pairing as observed by native DNA gel analyses (Fig. 3A). Moreover, DNase I protection analysis of RecA–ssDNA complexes formed on such repetitive versus mixed sequence templates did not reveal any intrinsic affinity differences in the binding of RecA under similar conditions (unpublished observations). Thereby, the slow decline in fluorescence reflects on the slow dissociation of RecA from these sub-optimally paired realignment intermediates, rather than the intrinsic affinity differences of the protein for such substrates. The slow kinetics of RecA fall off observed in this fluorescence assay mirrors the slow kinetics of repeat realignment revealed by TL assay [9]. These results therefore corroborate each other suggesting an active link between RecA status and strand realignment. However, such slow kinetics is not compatible with the expected rates in vivo. The in vitro reaction described here is mediated entirely by a single protein, RecA, whereas in vivo the same is likely to be assisted by a myriad of helper molecules such as RecO, RecR, SSB [29–31]. One must note that RecA fall off from these intermediates was not complete even after 5000 s ($\sim$80 min) as monitored by a RecA chase at step C (Fig. 5b). Hence, as contrasted by step C in Fig. 5c versus step B in Fig. 5b, one could surmise that RecA association is functionally linked to the corresponding realignment status of the duplexes. These results suggest the ability of RecA to sense sub-optimal alignments and linger on such complexes until realignment is complete. It is important to note that if stochastic pairings would lead to overhangs/gaps larger than five nucleotides, the 2AP probe situated towards the 3′/5′ end (fifth nucleotide from the end) would remain in an unpaired state thereby leading to higher steady state levels of fluorescence than compared to a probe at the center. However, different positions of 2AP did not show any effect either on the kinetics of pairing or the steady states of fluorescence (Fig. 5). Thus it seems that RecA is able to signal realignment in duplexes with gaps that are appreciably smaller and in fact probably lesser than even five nucleotides. What triggers RecA fall off from such maximized duplexes in spite of ssDNA levels almost comparable to that of the intermediates during realignment (compare (c) versus (a)/(b) in Fig. 1)? How does RecA sense the incompleteness of these alignments and subsequently fall off following the realignment of these duplexes?

Our recent observations suggest that RecA mediated realignment is intimately linked with the ongoing cycles of ATP hydrolysis, where an abrogation of hydrolysis (in ATPγS) leads to cessation of realignment activity [9]. What is the relationship between the compact/extended helix of the RecA filament versus the dynamic changes associated with the DNA bases during structural transitions that ensue during cycles of ATP hydrolysis? Nishinaka et al. have suggested that an inter-conversion of the backbone sugar puckers mediates translational and rotational motion of the bases in an active RecA filament coupled with cycles of ATP–ADP exchange [12]. We hypothesize that the enhanced fluidity observed across repeat DNA stretches may stem from such inter-conversions and in turn allow a non-planar H bonding geometry across the adjacent stacks thus mediating a dynamic 'cross talk' (Fig. 6). In fact, crystal structure of poly(dA):poly(dT) duplexes indicates that a high propeller twist of the bases sets up a continuous bifurcating hydrogen bonding system along the major groove of the helix [32,33], which probably underlies the dynamic cross talk in an active RecA filament. As described in the model (Fig. 6), we believe that a cross talk at monomer I ultimately succeeds in stabilizing a new base pair at monomer III by a concerted/cumulative action of all intervening A:T cross talks, which might result in waves of helix distortions as a function of the progressive cross talk. A higher affinity of RecA for such helical distortions in the filament [34] might help retain the protein on these realignment intermediates. One could envisage this process recurring until all unpaired A and T bases in sub-optimally aligned complexes are fully paired leading to a maximization of homologous alignment, thereafter relieving distortions in the helix and triggering RecA dissociation. Thus, RecA is able to persist on such intermediates, as long as it is able to mediate an efficient ATP hydrolysis coupled with a cross talk across the bases, and falls off, marking the completion of realignment.

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References

[1] Radding, C.M. (1991) J. Biol. Chem. 266, 5355–5358.
[2] Kowalczykowski, S.C. and Eggleston, A.K. (1994) Annu. Rev. Biochem. 63, 991–1043.
[3] Radding, C.M. (1993) Curr. Biol. 3, 358–360.
[4] Cox, M.M. (1993) Bioessays 15, 617–623.
[5] Roca, A.I. and Cox, M.M. (1997) Prog. Nucleic Acid Res. Mol. Biol. 56, 129–223.
[6] Camerini-Otero, R.D. and Hsieh, P. (1995) Annu. Rev. Genet. 29, 509–552.
[7] Jeffreys, A.J., Barber, R., Bois, P., Buard, J., Dubrova, Y.E., Grant, G., Hollies, C.R., May, C.A., Neumann, R., Panayi, M., Ritchie, A.E., Shone, A.C., Signer, E., Stead, J.D. and Tamaki, K. (1999) Electrophoresis 20, 1665–1675.
[8] Epplen, C., Santos, E.J., Maueler, W., van Helden, P. and Epplen, J.T. (1997) Electrophoresis 18, 1577–1585.
[9] Sen, S., Karthikeyan, G. and Rao, B.J. (2000) Biochemistry 39, 10196–10206.
[10] Dutreix, M. (1997) J. Mol. Biol. 273, 105–113.
[11] Ogawa, T., Yu, X., Shinohara, A. and Egelman, E.H. (1993) Science 259, 1896–1899.
[12] Nishinaka, T., Shinohara, A., Ito, Y., Yokoyama, S. and Shibata, T. (1998) Proc. Natl. Acad. Sci. USA 95, 11071–11076.
[13] Shibata, T., Cunningham, R.P. and Radding, C.M. (1981) J. Biol. Chem. 256, 7557–7564.
[14] Karthikeyan, G., Wagle, M.D. and Rao, B.J. (1998) FEBS Lett. 425, 45–51.
[15] Menetski, J.P. and Kowalczykowski, S.C. (1987) J. Biol. Chem. 262, 2093–2100.
[16] Menetski, J.P. and Kowalczykowski, S.C. (1987) J. Biol. Chem. 262, 2085–2092.
[17] Ward, D.C., Reich, E. and Stryer, L. (1969) J. Biol. Chem. 244, 1228–1237.
[18] Allan, B.W., Beechem, J.M., Lindstrom, W.M. and Reich, N.O. (1998) J. Biol. Chem. 273, 2368–2373.
[19] Holz, B., Klimasauskas, S., Serva, S. and Weinhold, E. (1998) Nucleic Acids Res. 26, 1076–1083.
[20] Lakshmikanth, G.S. and Krishnamoorthy, G. (1999) Biophys. J. 77, 1100–1106.

[21] Hochstrasser, R.A., Carver, T.E., Sowers, L.C. and Millar, D.P. (1994) Biochemistry 33, 11971–11979.
[22] Aymami, J., Coll, M., Frederick, C.A., Wang, A.H. and Rich, A. (1989) Nucleic Acids Res. 17, 3229–3245.
[23] Stasiak, A. and Egelman, H. (1988) in: Genetic Recombination (Kucherlapati, R. and Smith, R.G., Eds.), pp. 265–308, American Society for Microbiology, Washington, DC.
[24] Muller, B. and Stasiak, A. (1991) J. Mol. Biol. 221, 131–145.
[25] Chow, S.A., Honigberg, S.M., Bainton, R.J. and Radding, C.M. (1986) J. Biol. Chem. 261, 6961–6971.
[26] Millar, D.P. (1996) Curr. Opin. Struct. Biol. 6, 322–326.
[27] Lam, W.C., Van der Schans, E.J., Sowers, L.C. and Millar, D.P. (1999) Biochemistry 38, 2661–2668.
[28] Allan, B.W., Reich, N.O. and Beechem, J.M. (1999) Biochemistry 38, 5308–5314.
[29] Harmon, F.G. and Kowalczykowski, S.C. (1998) Genes Dev. 12, 1134–1144.
[30] Shan, Q., Bork, J.M., Webb, B.L., Inman, R.B. and Cox, M.M. (1997) J. Mol. Biol. 265, 519–540.
[31] Webb, B.L., Cox, M.M. and Inman, R.B. (1997) Cell 91, 347–356.
[32] Coll, M., Frederick, C.A., Wang, A.H. and Rich, A. (1987) Proc. Natl. Acad. Sci. USA 84, 8385–8389.
[33] Nelson, H.C., Finch, J.T., Luisi, B.F. and Klug, A. (1987) Nature 330, 221–226.
[34] Wang, Y.H., Bortner, C.D. and Griffith, J. (1993) J. Biol. Chem. 268, 17571–17577.