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The ATPase activity of RecA is needed to push the DNA strand exchange through heterologous regions

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The role of ATP hydrolysis during the RecA-mediated recombination reaction is addressed in this paper. Recent studies indicated that the RecA-promoted DNA strand exchange between completely homologous double- and single-stranded DNA can be very efficient in the absence of ATP hydrolysis. In this work we demonstrate that the energy derived from the ATP hydrolysis is strictly needed to drive the DNA strand exchange through the regions where the interacting DNA molecules are not in a homologous register. Therefore, in addition to the role of the ATP hydrolysis in promoting the dissociation of RecA from the products of the recombination reaction, as described earlier, ATP hydrolysis also plays a crucial role in the actual process of strand exchange, provided that the lack of homologous register obstructs the process of branch migration.

Key words: ATP hydrolysis/recombination/strand exchange

Introduction

In the standard *in vitro* recombination reactions, RecA hydrolyzes ATP during all sequential stages of the reactions (for recent reviews discussing the ATPase function of RecA see: Kowalczykowski, 1987, 1991; Roca and Cox, 1990; Stasiak *et al.*, 1991). The ATPase activity of RecA is induced as soon as RecA binds to single- or double-stranded DNA and forms the so called presynaptic complexes. The hydrolysis of ATP continues with an almost constant speed during the search for homology between the interacting DNA molecules and later during the stage of the reaction where the strands are exchanged between the paired DNA molecules. The omnipresent ATPase activity of RecA led to the proposal that the energy derived from the ATP hydrolysis is required for the search for homology (Cox *et al.*, 1987) and for the actual process of strand exchange (Cox and Lehman, 1981; Riddles and Lehman, 1985). However, more recent studies in several laboratories have demonstrated that RecA is not only able to mediate the specific homologous recognition between the interacting DNA molecules in the absence of ATP hydrolysis (Honigberg *et al.*, 1985; Müller *et al.*, 1990), but can also mediate the actual process of strand exchange between completely homologous DNA molecules (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990). It was demonstrated that in reactions between completely homologous DNA molecules, ATP hydrolysis was only required for the very last stage of the reaction, namely the dissociation of RecA from the DNA products of recombination (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990; Stasiak *et al.*, 1991).

The question arising was: why is RecA so reckless with the ATP hydrolysis, when it would be much wiser to have the ATPase activity being induced after the strands are exchanged? If that were the case, ATP could be saved and only used for the dissociation of RecA from the DNA products of recombination.

We decided to test if hydrolysis of ATP is necessary in a specific situation such as in the presence of heterologous sequences in the recombining molecules. We noticed that in these reactions ATP hydrolysis is strictly required to allow RecA to push the DNA strand exchange through the regions where the interacting molecules are not homologously aligned. Since natural recombination reactions frequently deal with stretches of heterologous sequence, this could explain why RecA evolved in such a way that it mobilizes the ATPase activity during the entire process of the strand exchange.

Results

Strand exchange reactions performed in the presence of ATP are reversible and this lowers the yield of these reactions

The best characterized type of reaction catalyzed by RecA is the non-reciprocal strand exchange between double-stranded (ds) and single-stranded (ss) DNA molecules. In these reactions, the single-stranded DNA partner replaces one strand of the interacting duplex DNA. It is well known that these reactions can occur between fully base-paired duplex and completely or only partially homologous single-stranded DNA molecules (Bianchi and Radding, 1983). We decided to compare these standard reactions with reactions where bulged duplex DNA molecules were provided instead of fully base-paired duplexes. For our reactions we used synthetic ss and ds oligonucleotides where the individual strands ranged from 48 to 60 nucleotides. Oligonucleotides of this size are convenient test substrates for RecA-mediated reactions, since DNA molecules of this length support the formation of stable presynaptic complexes and are long enough to be homologously recognized by RecA (Rosselli and Stasiak, 1990).

Figure 1 shows an autoradiogram of a gel with the samples of three different strand exchange reactions occurring between double- and single-stranded oligonucleotides. The drawings at the top of the gel explain how specific strand labeling was used to monitor the progress of these reactions. The first reaction (lane 2) occurs between completely homologous partners: a 54 bp duplex (ds54mer) and a 54 nucleotide single-stranded DNA (ss54mer). The occurrence of this reaction is manifested in this case by the release of a labeled single strand from the duplex DNA molecule. In the second reaction (lane 4) the duplex substrate (ds54mer) is the same as in the first reaction but the homologous ss54mer is replaced by a 60mer with a

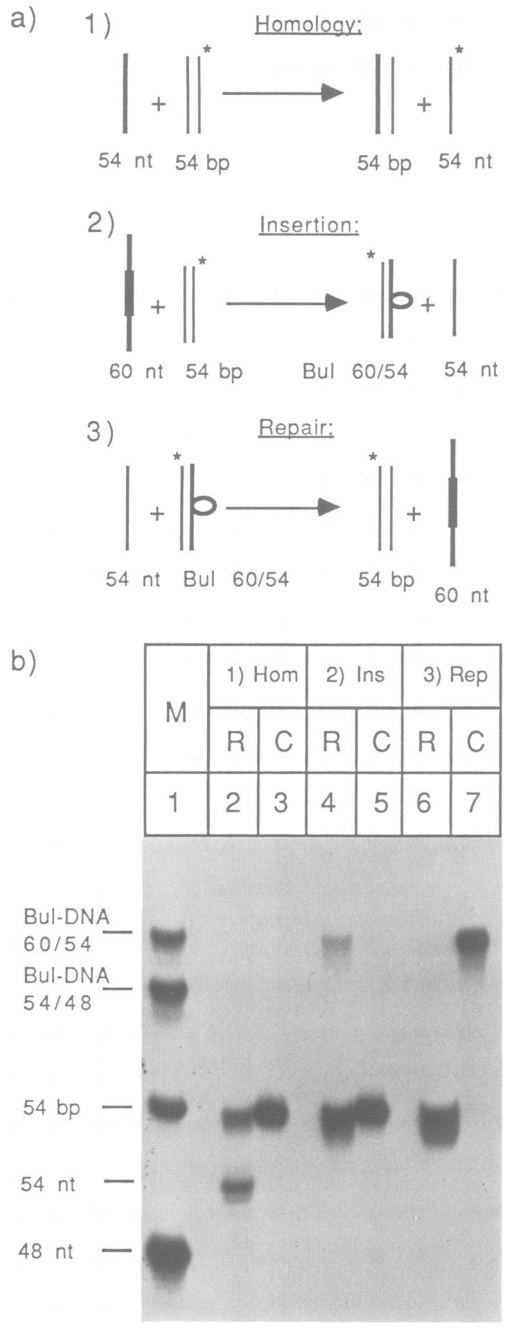

Fig. 1. Comparison between three different types of strand exchange reactions performed in the presence of ATP. (**a**) Schematic drawings of the reactions; (**b**) Autoradiogram of the dried 12% polyacrylamide, native gel analyzing the reactions presented in (a). Lane 1: marker DNAs; the length in nucleotides or base-pairs is indicated at the side. Lane 2: strand transfer reaction between completely homologous DNA molecules (drawing 1). Lane 4: reaction between partially heterologous DNA molecules leading to the creation of a bulged heteroduplex (drawing 2). Lane 6: correction of the bulged heteroduplex into a fully base-paired duplex, (drawing 3). Lanes 3, 5 and 7: control reactions corresponding to lanes 2, 4 and 6 respectively, lacking RecA protein in the reaction mixture.

heterologous insertion of six nucleotides placed in the center of the strand. The occurrence of this reaction can be followed by the conversion of the labeled homoduplex into a bulged duplex showing reduced electrophoretic mobility (Bhattacharya and Lilley, 1989). In the third reaction (lane 6), the substrates are identical to the products of the second reaction. The progress

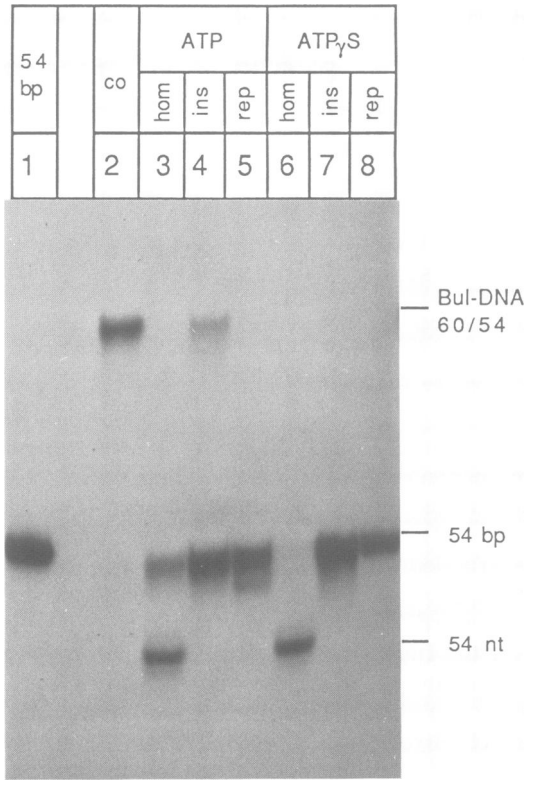

Fig. 2. Comparison between reactions performed in the presence of ATP or ATP-γ-S. The outcome of the three different strand exchange reactions presented in Figure 1 are analyzed here after being performed in the presence of ATP (lanes 3, 4 and 5) or ATP-γ-S (lanes 6, 7 and 8). Lane 1 shows the position of the ds54mer. Lane 2: control reaction for 'repair' in the absence of RecA protein.

of this reaction is evident from the complete conversion of the bulged duplex into a fully base-paired duplex (the opposite of the second reaction).

As seen in Figure 1, in the presence of ATP RecA is able to promote a strand exchange reaction between completely homologous ss54mer and ds54mer and between partially heterologous ss60mer and ds54mer. The efficiency of the heterologous reaction is much lower than that of the homologous reaction, but even in the homologous reaction the efficiency does not seem to reach more than 50% after 30 min of reaction. This observation is consistent with out earlier finding, that in the presence of ATP, the strand exchange between fully homologous double- and single-stranded oligonucleotides can reach an equilibrium state between forward and backward reactions within a few minutes (Rosselli and Stasiak, 1990). Since in the reactions between completely homologous substrates the forward and the backward reactions have similar speed, the apparent yield of the whole reaction at equilibrium depends on the input ratio between the substrate single- and double-stranded DNA. In the case where the input ratio of ssDNA molecules to dsDNA molecules is 1:1, the maximal expected efficiency at the equilibrium can only reach 50%. The situation is different in the case of a reaction between partially heterologous substrates, where it is known that the strand exchange is slower than the exchange between homologous strands (Bianchi and Radding, 1983). Interestingly, in the reaction analyzed in lane 4, only the forward reaction has to traverse a stretch of heterology. In the backward reaction, where the bulged duplex is corrected to completely base-paired duplex,

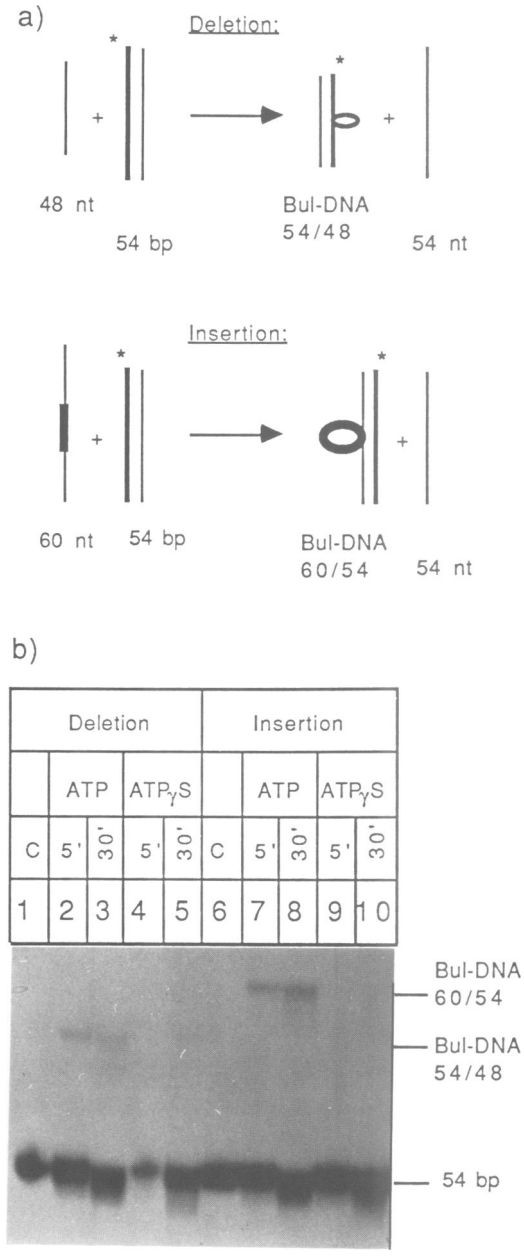

Fig. 3. The ATPase activity of RecA is needed to push the strand exchange through a heterologous insertion in the single- or double-stranded partner. (**a**) Schematic drawings of the two partially heterologous reactions analyzed in lanes 2–5 and 7–10 respectively. (**b**) Lanes 2, 3 and 7, 8 analyze reactions performed in the presence of ATP. The appearance of labeled molecules with characteristic migration of bulged molecules indicates that in both these reactions the strand exchange traverses a stretch of heterology. The lack of labeled bulged molecules in lanes 4 and 5, and 9 and 10 demonstrates that, in the presence of ATP-γ-S, the strand exchange is not able to traverse a heterologous insertion in the single- or double-stranded partner. Lanes 1 and 6 analyze the corresponding control reactions performed in the absence of RecA.

the exchanged strand does not encounter any obstacles during its complete switch between the two partners. Therefore, being more efficient, the backward reaction can cancel the effect of the forward reaction. Consequently, the apparent total yield of the heterologous reactions (like the one analyzed in lane 4) rather under-represents the ability of RecA to mediate the strand exchange between partially heterologous DNA molecules.

The opposite situation can be observed in the reactions analyzed in lane 6, where the forward reaction does not need to traverse any region of heterology, while the backward does. This situation leads to a very high apparent total yield of such a strand exchange reaction.

ATP hydrolysis is needed to push the strand exchange through heterologous regions

We showed earlier that, in the presence of ATP-γ-S, RecA can promote a very efficient strand exchange between completely homologous ds and ss oligonucleotides (Rosselli and Stasiak, 1990). However, the lack of ATP hydrolysis leads to a stable association between RecA and the DNA products of the strand exchange. Therefore, to observe the products of the reaction it is necessary to dissociate RecA from the DNA before analyzing them on the gel. Since the DNA products of the forward reaction are precluded from undergoing the backward reaction, this situation leads to the accumulation of the DNA products of the first round. Therefore, the strand exchange between completely homologous ss and ds DNA molecules performed in the presence of ATP-γ-S has very high efficiency (Rosselli and Stasiak, 1990; Stasiak *et al.*, 1991).

Figure 2 nicely illustrates this energetic aspect of RecA-mediated DNA strand exchange. It is clearly visible that the strand exchange between homologous ss and ds DNA molecules leads to a balanced equilibrium in the case of the reaction performed in the presence of ATP (lane 3). However, the same reaction performed in the presence of ATP-γ-S leads to the complete displacement of the labeled strand of the substrate duplex (lane 6). The reactions analyzed in lanes 5 and 8 demonstrate that in the reaction performed in the presence of ATP, when the forward direction does not need to traverse a segment of heterology but the reverse direction does, the total yield of such reactions is as high as that of the reactions performed in the presence of ATP-γ-S. We interpret this as a nice demonstration that, when in the ATP-stimulated reaction the backward reaction is much slower than the forward one, such a situation resembles the effect of ATP-γ-S, which blocks the backward reaction.

Since the strand exchange reactions performed in the presence of ATP-γ-S are irreversible, one could in principle expect these reactions to be very efficient in promoting reactions through heterology since the reverse reaction would not cancel the effect of the forward reaction. That was clearly not what we observed. While the heterologous reaction performed in the presence of ATP (lane 4) gave a typical low yield (diminished by backward reaction), the reaction performed in the presence of ATP-γ-S gave almost no yield at all (lane 7). Figure 2 demonstrates therefore, that the hydrolysis of ATP is required for strand exchange between partially heterologous sequences. It seems that ATP hydrolysis is only needed to push the strand exchange through the regions of heterology since the homologous reaction performed in the presence of ATP-γ-S was much more efficient than in the presence of ATP (compare lanes 3 and 6 and 4 and 7). To demonstrate more convincingly the requirement for ATP hydrolysis to drive the strand exchange through heterologous regions, we decided to test some other reactions between partially heterologous substrates. In the reaction analyzed in Figure 2, the single-stranded DNA substrate had a six nucleotide heterologous insertion in relation to the duplex substrate. We decided to compare this

reaction with one in which the ss DNA substrate had a six nucleotide deletion in relation to the duplex substrate. As shown in Figure 3, both heterologous reactions required ATP to obtain a significant amount of the product.

The strand exchange can bypass a short heterology in the absence of ATP hydrolysis if the heterologous region does not break the homologous register between the two paired molecules

During the strand exchange reaction between homologous DNA molecules, the two interacting DNA molecules are brought into homologous, coaxial alignment within the RecA helical filaments (Howard-Flanders *et al.*, 1984; Stasiak *et al.*, 1984; Christiansen and Griffith, 1986; Register *et al.*, 1987). Therefore, for every base-pair opened in a substrate duplex, a new one can be formed in a product duplex. This leads to an isoenergeticity of the strand exchange and explains why ATP hydrolysis is not necessary for the actual exchange between homologous DNA molecules (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990). In the case of partially heterologous DNA molecules, the homologous register of the two molecules acheived in front of heterologous region has to be broken at the region of heterology. We propose that behind the region of heterology, the interacting molecules may remain out of homologous register even when the heterology is as small as a deletion of 1 bp. In such a case, starting from the region of heterology, RecA would have to open the base pairs of a substrate duplex without the reforming of base-pairing in a product duplex. This process would certainly need a lot of energy, which could be provided by the ATPase activity of RecA. The annealing of the two strands of the nascent heteroduplex behind the region of heterology may be much later step of the reaction, so that the energy released during the annealing could not be coupled to the preceding process of strand separation.

We observed an ATP dependence of the strand exchange reaction where the single-stranded DNA had a six nucleotide heterologous insertion in relation to the duplex substrate (Figures 2 and 3). Since the number of paired bases is not changed and the free energy of the bulged duplex is not much higher than that of a regular duplex, one could expect such a reaction to proceed in the absence of ATP hydrolysis. However, if the two interacting DNA molecules are not in homologous register behind the heterologous insertion, there is a need to provide some energy to separate the strands of duplex until the end of the duplex DNA molecule or until the establishment of the homologous register. Careful analysis of the experiments performed by Bianchi and Radding (1983) reveals that the efficiency of strand exchange does not decrease significantly when the heterologous insertion in the substrate single-stranded DNA is increased from 33 to 1308 nucleotides. This result supports our proposal that the effect of small or large heterology can be the same, since in any case the interacting DNA molecules are out of the homologous register from the point where the heterology starts.

We therefore decided to test if we could allow the strand exchange reaction to proceed in the absence of ATP hydrolysis by bringing a homologous register behind a short heterology. To meet this aim we performed a strand exchange reaction in which the bulge was conserved since the displaced short strand was replaced by an identical strand

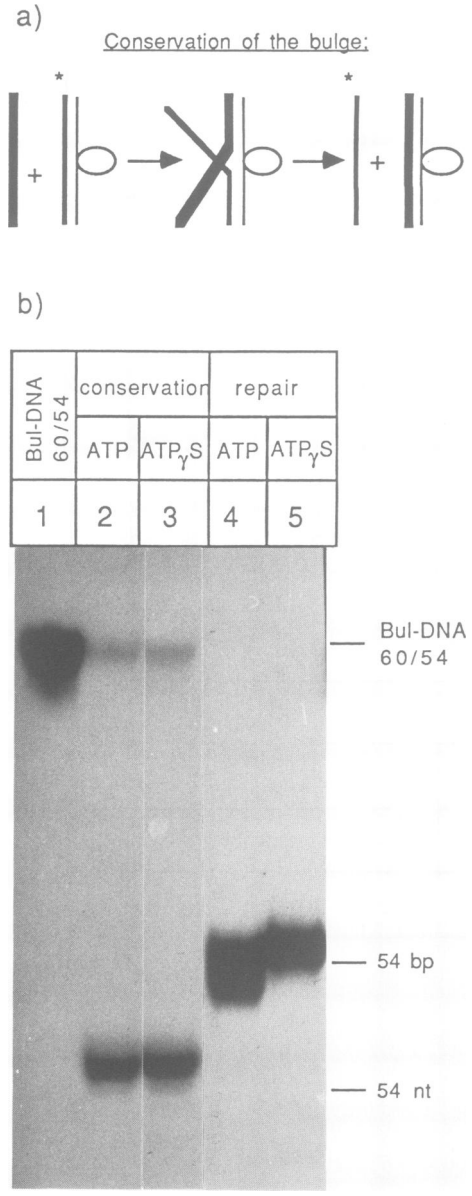

Fig. 4. Introducing the homologous register after a short heterology restores the ATP independence of the strand exchange process. (**a**) Schematic drawing of the reaction. The labeled substrate is a slowly migrating bulged molecule. The labeled product is a quickly migrating displaced strand. (**b**) Autoradiogram showing that this type of heterologous reaction proceeds with at least the same efficiency in the presence of ATP-γ-S (lane 3) as in the presence of ATP (lane 2). Lane 1 shows the migration of the labeled bulged DNA. The apparent high efficiency of the reaction performed in the presence of ATP can result from the high excess of unlabeled substrate ssDNA used. Lanes 4 and 5: as a comparison, repair reaction leading to the conversion of the labeled bulged DNA into completely double-stranded 54mer DNA.

(see drawing at the top of Figure 4). Therefore, during the strand exchange, the incoming single strand has to bridge-over the heterologous insertion in the same way as the displaced strand. As seen in Figure 4, the reaction performed in the presence of ATP-γ-S was at least as efficient as the reaction performed in the presence of ATP. We interpret this result as an indication that the strands to be paired were in a homologous register before and after the heterologous insertion. Bulged DNA molecules can be predestined for such an alignment since the heterologous bulge can be looped

out and the shorter strand determines the end-to-end length of the whole molecule (Bhattacharyya and Lilley, 1989). Therefore, in the RecA synaptic filaments, the single-stranded substrate and the interacting bulged molecule can occupy the same stretch within the complex. As a result, the strand exchange between such partially heterologous molecules could proceed without the need for ATP hydrolysis, since the molecules are in the homologous register over their entire length, so that for every base pair opened a new one can be immediately reformed.

Discussion

Our experiments demonstrate that the ATPase activity of RecA is required for the process of strand exchange to allow the branch migration to traverse regions of non-homology. This observation nicely complements earlier findings that RecA can promote strand exchange between completely homologous DNA molecules in the absence of ATP hydrolysis (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990). DNA strand exchange between homologous DNA molecules is a kind of isoenergetic reaction, where for every base-pair opened a new one is formed (Kowalczykowski, 1987, 1991; Roca and Cox, 1990; Cox, 1991; Stasiak *et al.*, 1991). In a reaction between homologous DNA molecules the energy may only be needed to shift the equilibrium towards the products. It was proposed that the energy of the complex formation between RecA and DNA can be used to push the reaction to completion (Rosselli and Stasiak, 1990). This can be achieved since in the absence of ATPase activity, RecA presumably forms tighter complexes with the products than with the substrates of the reaction (Kowalczykowski, 1987, 1991; Stasiak *et al.*, 1991). Therefore, for strand exchange between completely homologous DNA molecules, ATP hydrolysis is only required for the dissociation of the post-exchange RecA–DNA complexes (Rosselli and Stasiak, 1991).

During the strand exchange reaction between partially heterologous DNA molecules, not every base-pair opened is compensated by a new base-pair formed. It was earlier elegantly demonstrated that RecA-mediated strand exchange can traverse kilobase-long heterologies (Bianchi and Radding, 1983). When the substrate duplex DNA contained a long insertion in relation to the interacting single-stranded DNA, then during the reaction the region of this insertion had to be promptly strand-separated without compensatory base-pair formation. It is obvious that such a strand separation requires a lot of energy. We propose that the ATPase activity of RecA provides the energy needed to separate the strands of those regions of duplex DNA which are not in homologous alignment with the interacting partner.

Why is RecA hydrolyzing ATP during the entire process of strand exchange?

If one accepts that ATP hydrolysis is needed for the process of strand exchange between partially heterologous sequences and for the dissociation of RecA from the DNA products of recombination, one has to ask why the ATPase activity of RecA is already induced before the strand exchange reaches the first heterology. One possible answer is that the biological sense of the recombination reaction is achieved only when the displacing strand is not exactly identical to the displaced strand. Most likely, in the *in vivo* situation, the exchanged strands frequently have to traverse some regions of heterology. We indicated here that even a small heterology like a short deletion or insertion can put two molecules out of homologous register, so that the processing strand exchange needs the energy of ATP hydrolysis to compensate for the base-pairing left open behind the heterologous region. Our recent observations also indicate that the strand exchange reaction between two double-stranded DNA molecules requires ATP hydrolysis even if proceeding through regions of homology (W.Rosselli and A.Stasiak, unpublished data). Since RecA in the biologically relevant strand exchange reactions has to deal with heterologies and have to be able to exchange strands between two double-stranded DNA regions, this explains why RecA evolved this way so that it mobilizes its ATPase activity even if promoting a strand exchange between completely homologous DNA molecules. It has to be realized that, even in the case of completely homologous partners, as soon as the strands get exchanged the ATPase activity is needed for the release of the DNA products and the recycling of RecA (Rosselli and Stasiak, 1990), therefore, the ATPase activity of RecA should be associated all the time with progressing strand exchange. In addition it might turn out that the ATPase activity of RecA speeds up the process of homologous recognition or strand exchange between completely homologous DNA molecules although it was demonstrated that these processes can occur quite efficiently in the absence of ATP hydrolysis (Honigberg *et al.*, 1985; Müller *et al.*, 1990; Menetski *et al.*, 1990; Rosselli and Stasiak, 1990).

Materials and methods

RecA protein

RecA protein was isolated from the *Escherichia coli* strain KM4104 transformed with the plasmid pDR1453 (Sancar and Rupp, 1979). RecA protein was purified by the procedure described by Cox *et al.* (1981) to fraction III. The concentration was determined using a $E_{277}1\%$ of 6.33, according to Tsang *et al.* (1985).

DNA substrates

The gel-purified oligonucleotides were purchased from MedProbe (Norway). For completely homologous strand exchange reactions we have chosen oligonucleotides with a natural sequence from phage ΦX174 DNA, 5′-TACGTTAACAAAAAGTCAGATATGGACCTTGCTGCTAAAGG-TCTAGGAGCTAAA-3′ and the complementary oligonucleotide 5′-AGCT-CCTAGACCTTTAGCAGCAAGGTCCATATCTGACTTTTTGTTAA-CGTATTT-3′. The reaction occurred between the annealed duplex and one of the single strands. For partially heterologous reactions the annealed duplex was reacted with a strand having either a six nucleotide heterologous insertion or six nucleotides deleted in the center of the strand. The double-stranded 54mers were produced by annealing of the two strands in 150 mM NaCl and 15 mM sodium by heating to 55°C for 30 min followed by slow cooling to room temperature. Bulged molecules were also prepared by annealing of partially heterologous oligonucleotides. After annealing, duplex or bulged DNA molecules were separated by non-denaturating polyacrylamide gel electrophoresis from possible single-stranded contamination. After separation, the DNA was visualized by ethidium bromide staining or by directly exposing the gel on an X-ray film (in the case of radioactively labeled DNA), the band of the duplex or bulged DNA was cut out and the DNA was eluted from the gel and purified from the residual polyacrylamide and ethidium bromide (in the case of non-labeled DNA).

5′-end labeling of the 54mers was performed at the level of single-stranded DNA using T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]ATP (Amersham). Labeled duplex 54mers contained only one labeled strand and were produced by annealing of the labeled strand with the complementary non-labeled strand, with subsequent gel purification.

The concentrations of DNA were determined spectrophotometrically using $1OD_{260}$ = 36 μg/ml for ssDNA and $1OD_{260}$ = 50 μg/ml for dsDNA. The DNA concentrations are expressed as concentration of DNA nucleotides.

DNA strand exchange assays

Pre-incubation of the mixture of 25 mM TEA-acetate (pH 6.8), 20 mM phosphocreatine, 10 U/ml creatine kinase, 8 μM RecA, 25 μM ssDNA, 2 mM Mg-acetate and 2 mM ATP or 2 mM ATP-γ-S (when ATP-γ-S was used the ATP regeneration system was omitted) for 5 min at 37°C. Subsequently 12.5 μM ^{32}P-labeled DNA (completely double-stranded or bulged duplex DNA) was added with a simultaneous raise in the concentration of Mg^{2}+ to 5 mM. The incubation continued at 37°C for 5–30 min and the reaction was stopped with 0.5% SDS and analyzed on a non-denaturating 12% polyacrylamide gel.

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Note added in proof

After this work had been completed, we became aware that the group of Professor M.M.Cox had independently demonstrated the requirement for ATP hydrolysis to promote the RecA-mediated recombination between partially heterologous DNA molecules (unpublished).