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Review Article

RecA and DNA recombination: a review of molecular mechanisms

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Recombinases are responsible for homologous recombination and maintenance of genome integrity. In *Escherichia coli*, the recombinase RecA forms a nucleoprotein filament with the ssDNA present at a DNA break and searches for a homologous dsDNA to use as a template for break repair. During the first step of this process, the ssDNA is bound to RecA and stretched into a Watson–Crick base-paired triplet conformation. The RecA nucleoprotein filament also contains ATP and Mg^{2+}, two cofactors required for RecA activity. Then, the complex starts a homology search by interacting with and stretching dsDNA. Thanks to supercoiling, intersegment sampling and RecA clustering, a genome-wide homology search takes place at a relevant metabolic timescale. When a region of homology 8–20 base pairs in length is found and stabilized, DNA strand exchange proceeds, forming a heteroduplex complex that is resolved through a combination of DNA synthesis, ligation and resolution. RecA activities can take place without ATP hydrolysis, but this latter activity is necessary to improve and accelerate the process. Protein flexibility and monomer–monomer interactions are fundamental for RecA activity, which functions cooperatively. A structure/function relationship analysis suggests that the recombinogenic activity can be improved and that recombinases have an inherently large recombination potential. Understanding this relationship is essential for designing RecA derivatives with enhanced activity for biotechnology applications. For example, this protein is a major actor in the recombinase polymerase isothermal amplification (RPA) used in point-of-care diagnostics.

Introduction

Homologous recombination is a mechanism that maintains the genomic integrity of all organisms by repairing DNA lesions, such as double-strand breaks (DSBs) and collapsed replication forks. The single-strand DNA (ssDNA) tracts formed at break sites are used to search for a homologous double-strand DNA (dsDNA) to use as a template to repair the break. This homology-driven mechanism is catalyzed by a class of DNA strand exchange proteins named recombinases [1].

As recombinases are essential for cell survival, they are ubiquitous throughout phages, bacteria, eukaryotes and archaea. Examples include RecA in bacteria, RadA in archaea and Rad51 in eukaryotes, and the RecA analogue UvsX is found in bacteriophages [2]. Although these recombinases show differences in the N-terminal and C-terminal regions, they are globally well-conserved proteins. Bacterial RecA proteins have an average sequence conservation of 60–70% across the entire bacterial domain [3].

These enzymes bind to ssDNA, forming a right-handed helical nucleoprotein filament containing multiple RecA monomers, and to ATP molecules that activate the filament. ATP binds to each monomer at the monomer/monomer interface. The ATP-bound nucleoprotein filament captures a homologous dsDNA during a process called homology searching to produce a synaptic structure. Once a region of homology is found, the ssDNA strands on the homologous chromosome are exchanged, producing heteroduplex DNA (i.e. each single strand originates from a different

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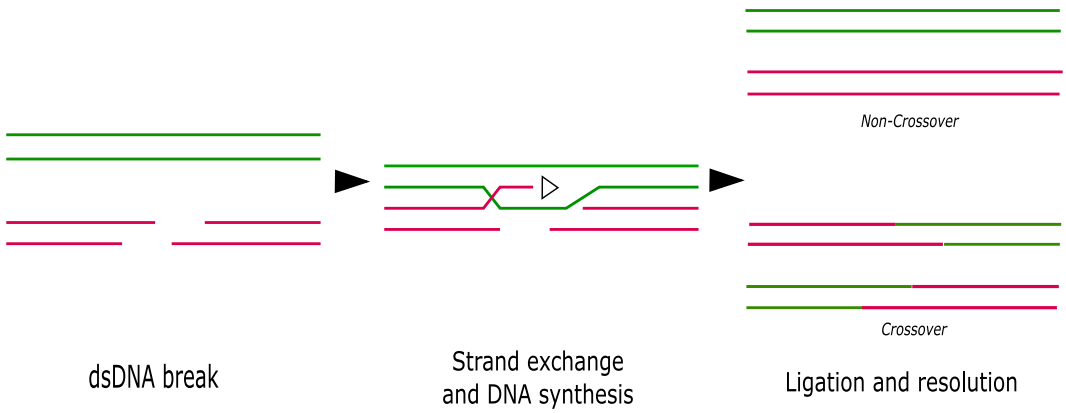

Figure 1. Schematic representation of homologous recombination.

The ssDNA tracts formed at a break site (in red) are used by the RecA protein to search for a homologous dsDNA (in green). The ssDNA–RecA filament performs a strand exchange between the ssDNA tract and the homologous dsDNA, which is then used as a template by the polymerases to synthesize DNA. This step is followed by ligation and resolution, which restore two dsDNA molecules (upper pathway) or generate two dsDNA molecules with crossovers (lower pathway).

chromosome). During this process, the filament presents an ATPase activity coupled to different steps of homologous recombination. Finally, a complex is produced that is an intermediate of recombination known as a heteroduplex joint. It is then repaired through a combination of DNA synthesis, ligation and resolution (Figures 1 and 2). Structurally, the homologous recombination implicates different domains of the RecA molecule: site I, where the ssDNA is bound initially and the heteroduplex joint is bound a little later in the process; site II, which interacts with the homologous dsDNA and the displaced strand; an ATP binding site and a hydrolysis motif responsible for the ATPase activity. Additionally, the N-terminal domain (NTD) and the C-terminal domain (CTD) interact with dsDNA during the homology search.

In vivo, many accessory proteins participate in recombination, composing a complex network of positive and negative effectors to precisely adapt the recombination activity to the cellular requirements. For example, in

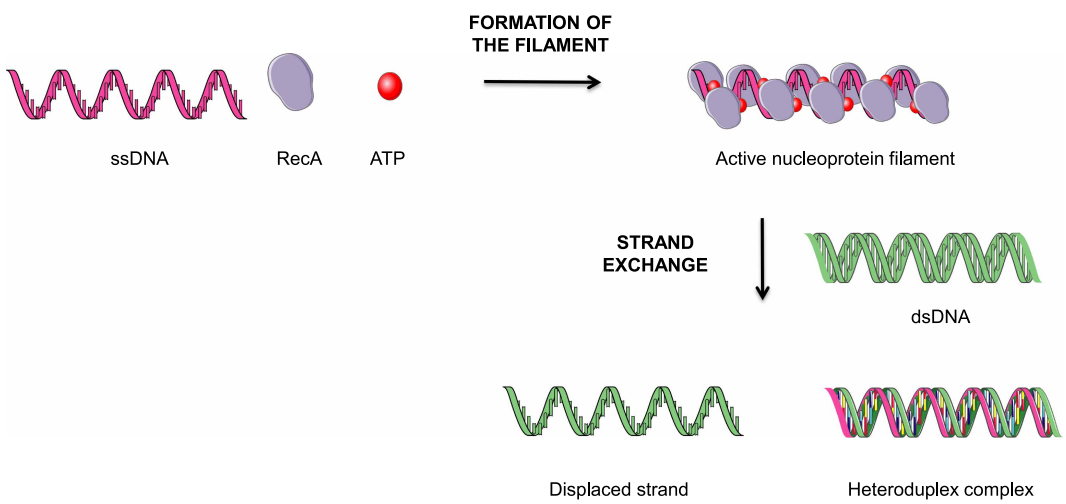

Figure 2. RecA activity illustration.

First, ssDNA (in pink) and RecA (in violet) and its cofactor ATP (in red) form an active right-handed helical nucleoprotein filament with six RecA monomers per turn. The filament further searches for and captures a homologous dsDNA (in green) to produce a synaptic structure. Once a region of homology is found, the ssDNA strands on the homologous chromosomes are exchanged, producing heteroduplex DNA.

Escherichia coli (*E. coli*), the single-stranded binding protein (SSB) coats and stabilizes ssDNA, denatures secondary structure that impedes nucleoprotein filament formation and stimulates DNA strand exchange by binding to the displaced ssDNA [4,5]. However, SSB can also inhibit RecA filament formation by competitive binding. Other examples of accessory proteins are RecFOR proteins, which have been shown to participate in the loading of RecA onto SSB-coated ssDNA; RecBCD, which process dsDNA breaks and directly load RecA onto ssDNA; and the RdgC, RecX proteins that inhibit RecA by blocking access to duplex DNA [6]. RecBCD is present mainly in the context of double-strand break repair (DSBR), and RecFOR is mainly present in the context of single-strand gap repair (SSGR) [7,8].

In addition to RecA activity being modulated by accessory proteins, RecA is also subject to autologous, intrinsic regulation: the 25 last amino acids of the CTD (called the C-tail) and some specific amino acids have a direct impact on the recombination function. These amino acids will be described later in the 'Hyper-recombinant forms of RecA' section. Therefore, RecA is subject to multiple layers of regulation and possesses an inherently large recombination potential. This potential can be characterized by the frequency of recombination exchanges (FRE) per DNA unit length. The FRE in some RecA mutants is increased up to 50-fold. RecA has not evolved to have an optimized activity but to preserve a balance between the different metabolic processes and genome stability. Actually, recombinogenic over-activity can have deleterious effects on the cell: in fact, non-controlled recombination can interfere with other biological processes, including DNA replication and transcription. Recombination can also cause genetic damage through the aberrant elimination of genomic segments by the recombination of repeat sequences [9]. Hyper-recombinogenic recombinases can be used *in vitro* for diagnostic applications, particularly in the context of isothermal amplification.

Currently, the technique most widely used to amplify nucleic acids and generate multiple copies of particular nucleic acid sequences is PCR. This technique requires constant changes in temperature and therefore the use of expensive equipment called thermocyclers. In contrast, isothermal amplification occurs at a constant temperature. This property alleviates the need for equipment, making diagnostics more affordable and easier to perform, even where the resources are limited. Furthermore, isothermal amplification techniques can be used in the context of point-of-care diagnosis while respecting the WHO (World Health Organization) ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable). Many isothermal amplification techniques have been developed; among these techniques, recombination polymerase amplification (RPA) uses a recombinase and a polymerase [10]. In fact, the recombinase strand exchange activity associated with its recombination function enables the recombinase to separate the two strands of the target dsDNA and fix ssDNA primers to it, empowering the polymerase to start amplification. This process is illustrated in Figure 3.

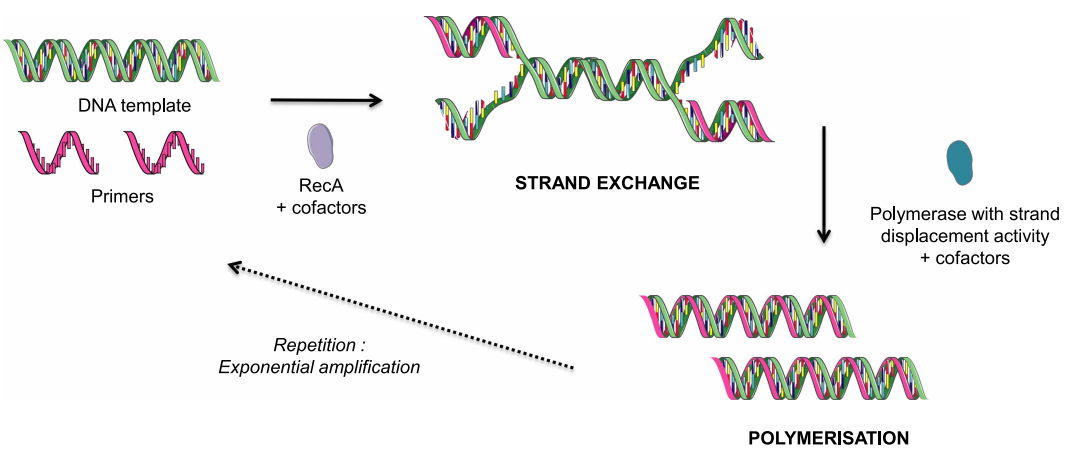

Figure 3. Recombinase polymerase amplification (RPA) illustration.

First, RecA (in violet) and its cofactors form a nucleoprotein filament with the single-strand primers (in pink) and fix them to the template, a homologous dsDNA (in green), using the strand exchange activity of the protein. A polymerase (in blue) with strand displacement activity that is accompanied by its cofactors is then able to polymerize DNA. The repetition of these steps results in an exponential amplification of the template DNA.

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This review focuses on the prototypic *E. coli* RecA recombinase and describes the relationship between its structure and its recombination-associated activities. In addition, a survey of hyper-recombination variants is presented. This information can be used to design an efficient recombinase in the context of isothermal amplification development.

Recombinases and structural organization

The first discovered and most studied RecA protein is *E. coli* RecA (EcRecA), which was discovered in 1965 by screening for mutants that had a deficient recombination function [11]. EcRecA is a 38 kDa protein composed of 352 amino acids and three major structural domains: a central core ATPase domain (CAD) and two smaller NTD and CTD [12] (Figure 4).

To complete its activities, RecA comprises four principal flexible regions: the region containing residues 24–37, which includes the end of the NTD and the beginning of the core domain; the loops L1 (residues 157–164) and L2 (residues 195–209); and finally the CTD, which is constituted by residues 270–352 [13] (Figure 4). Furthermore, the protein comprises different sites: an ATP binding site (residues 66–73); a ssDNA binding site (residues 157–216, also called site I); a dsDNA binding site (residues 226–245, also called site II); a domain (residues 270–328) that interacts with dsDNA called the dsDNA gateway and the C-terminus tail, which is composed of residues 329–352 and interacts with Mg^{2+} ions. Finally, residues 96 and 248–250 compose the catalytic region responsible for ATP hydrolysis. The Asp144 residue co-ordinates an Mg^{2+} ion at the active site between the β and γ phosphates of ATP [14]. These different features are recapitulated in a schematic representation of the one-dimensional structure of RecA (Figure 4).

The monomers form a polymer where they are arranged in a head-to-tail packing, with the positively charged surface of one monomer associated with the negatively charged surface of the neighbouring monomer, forming a contiguous helical polymer with an axial hole that accommodates at least three single strands of DNA. The contact interface of each subunit is composed of two regions: a first region, which consists of 14 residues located in positions 6–34 of the NTD, and a second region, which consists of 28 residues between positions 89 and 156 of the central domain and 13 residues between positions 172 and 257 of the central domain [12]. Different cross–subunit interactions between specific amino acids, such as the interactions between Lys6 and Asp139 side chains and between Arg28 and Asn113 side chains, have been revealed by mutation analysis. Additionally, largely hydrophobic interactions take place within these surfaces [15].

Figures 5 and 6 illustrate the alignment of the RecA sequences of different bacteria and other organisms to the EcRecA sequence to highlight the sequence conservation of the proteins belonging to this family, especially that of the core domain. Six bacterial species living in different environments were chosen (*Dickeya dadantii*, *Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Thauera hydrothermalis*, *Thauera aromatic* and *Deinococcus radiodurans*). The criterion of choice was their different living temperatures, so that RecA could be studied over a large temperature range, which is interesting for diagnostic applications. *Dickeya dadantii* lives in a plant environment with an optimal temperature of 30°C, *Pseudomonas fragi* is a psychrotrophic bacterial species,

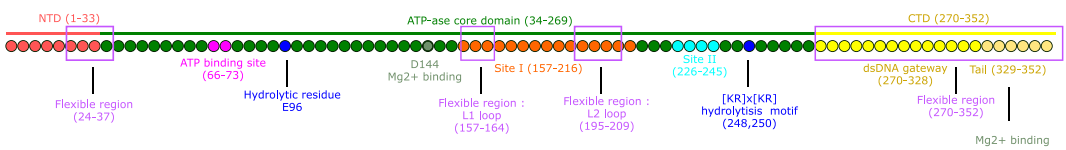

Figure 4. Schematic 1D RecA structure.

RecA is composed of 352 amino acids and three major structural domains: a central, core ATPase domain (CAD), which extends from the 34th to the 269th amino acid (in green), and two smaller NTD and CTD, which extend from the 1st to 33rd (in red) and from the 270th to 352nd amino acids, respectively (in yellow). The ATPase core domain includes different sites: the ATP binding site (residues 66–73, in pink); site I, which is the ssDNA binding site (residues 157–216, in orange); and the dsDNA binding site, site II (residues 226–245, in cyan). It also includes residues responsible for the ATP hydrolysis activity (E96 and the [KR] × [KR] motif in positions 248–250, in blue). The CTD can be divided into two domains: the dsDNA gateway (residues 270–328, in dark yellow) and the CTD tail (residues 329–325, in yellow) which modulates the protein activity. The Mg^{2+} binding is coordinated by D144 and CTD tail (in light green). Finally, the flexible regions of the protein are also depicted by violet boxes. These regions are the regions between residues 24 and 37 (at the end of the NTD and the beginning of the core domain), 157 and 164 (loop L1), 195 and 209 (loop L2) and 270 and 352 (CTD).

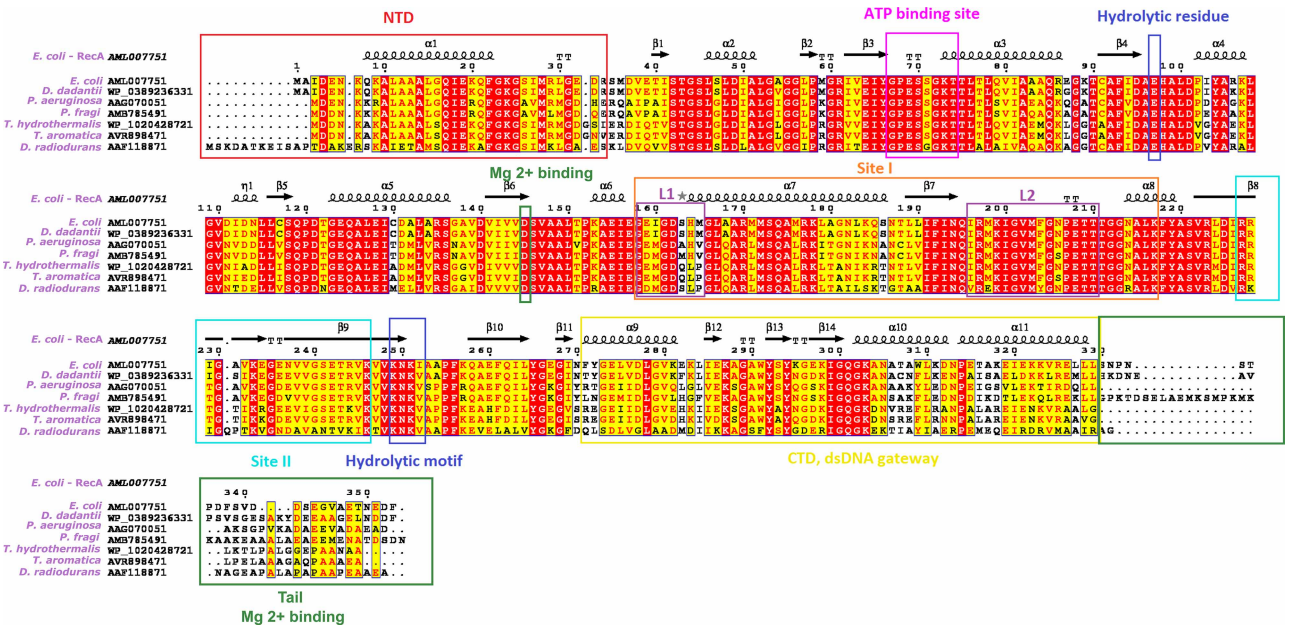

Figure 5. Multi-alignment of RecA from different bacteria.

The sequences aligned are from the following organisms. AML00775: *Escherichia coli* str. K-12 substr. MG1655 RecA; WP_038923633: *Dickeya dadantii* strain 3937 RecA; AAG07005: *Pseudomonas aeruginosa* PAO1 RecA; AMB78549: *Pseudomonas fragi* RecA; WP_102042872: *Thauera hydrothermalis* RecA; AVR89847: *Thauera aromatica* K172 RecA; and AAF11887: *Deinococcus radiodurans* R1 RecA. The filled red boxes indicate strict identity between residues, and the filled yellow boxes indicate strong homology between residues. No difference is made between black and red characters in this case because this feature is applied for other usages. At the top of the first sequence, the 2D structure of *E. coli* RecA is shown: the squiggles represent α-helices; the arrows, β-strands; the TT letters, strict β-turn; and the star, a residue with multiple conformations revealed by crystallography. The 2D structure is from the PDB entry 4TWZ. Additionally, the different domains of RecA are indicated in boxes: in red, the NTD; in pink, the ATP binding site; in green, the Mg^{2+} binding sites; in blue, the hydrolytic residue and the hydrolytic motif; in orange, site I; in cyan, site II; and in yellow, the dsDNA gateway in the CTD. The multi-alignment reveals a strong conservation of the RecA protein sequence among bacteria. The figure was generated using the ENDscript server [77].

Thauera hydrothermalis is thermophilic, and *Thauera aromatic* is a mesophilic bacterium. *Pseudomonas aeruginosa* and *Deinococcus radiodurans* have also been included in the analysis because of their development in extreme environments, which implicates hyper-recombinogenic activity: *P. aeruginosa* is subjected to high levels of oxidative damage and rapidly adapts to environmental changes, and *D. radiodurans* is well known for its capacity to survive extreme doses of ionizing radiation. Additionally, one organism per domain of life was chosen (the bacterium *E. coli*, the eukaryote *Homo sapiens* and the archaeon *Haloferax volcanii*; a bacteriophage, T4, was also included). The RecA proteins of different organisms have also been compared biochemically and mechanistically, evidencing that globally, even if some differences are present, the proteins function in a very similar way. Thus, *E. coli* RecA, bacteriophage T4 UvsX and *S. cerevisiae* Rad51 form similar nucleoprotein filaments and promote strand exchange in an analogous manner, surrounded by comparable accessory proteins. They differ in their NTP hydrolysis rates and requirements and in the polarity of DNA strand exchange (5′ → 3′ for RecA and UvsX, 3′ → 5′ for Rad51) [14].

The *P. aeruginosa* RecA protein called PaRecA is a protein 76% identical and 86% similar to EcRecA and presents an FRE that is 6.5 times greater than that of EcRecA [16]. Compared with EcRecA, PaRecA has a greater affinity for ssDNA, a more efficient SSB displacement, and a higher salt and temperature stability, and it disassembles at only half the rate of RecAEc protein filaments. The difference in the primary binding site affinity is caused by differences between positions 178 and 183 (L178I, A179T or L182I) (Figure 5) [17,18].

The *D. radiodurans* RecA protein called DrRecA is a protein 56% identical and 69% similar to EcRecA [19]. Thus, compared with EcRecA, DrRecA presents functional and structural differences. First, it uses an inverse pathway in which the filament is formed on dsDNA instead of ssDNA. [20] Then, it tends to create a large number of shorter filaments than EcRecA while it also nucleates more rapidly but extends the filaments more

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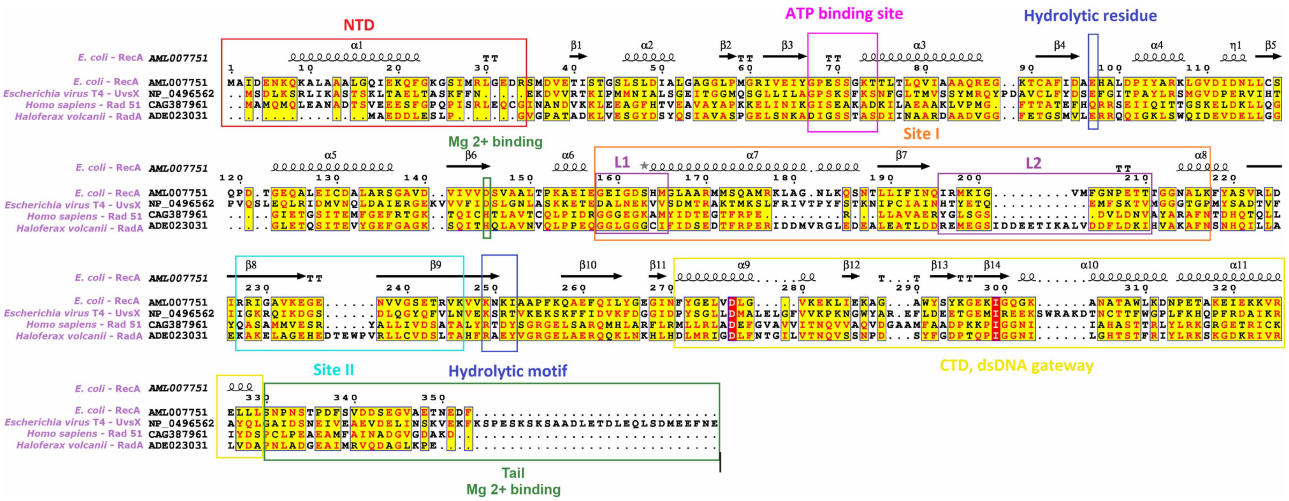

Figure 6. Multi-alignment of RecA proteins among organisms from different domains of life

The sequences aligned are from the following organisms. AML00775: *Escherichia coli* str. K-12 substr. MG1655 RecA; NP_049656.2 *Escherichia* virus T4 UvsX; CAG38796.1 *Homo sapiens* Rad51; and ADE02303: *Haloferax volcanii* DS2 RadA. The red boxes indicate strict identity between residues, and the yellow boxes indicate strong homology between residues. No difference is made between black and red characters in this case because this feature is applied for other usages. At the top of the first sequence, the 2D structure of *E. coli* RecA is shown: the squiggles represent α-helices; the arrows, β-strands; the TT letters, strict β-turns; and the star, a residue with multiple conformations revealed by crystallography. The 2D structure is from the PDB entry 4TWZ. Additionally, the different domains of RecA are indicated in boxes: in red, the NTD; in pink, the ATP binding site; in green, the Mg^{2+} binding sites; in blue, the hydrolytic residue and the hydrolytic motif; in orange, site I; in cyan, site II; and in yellow, the dsDNA gateway in the CTD. The multi-alignment reveals a conservation of the RecA protein sequence among phages, bacteria, eukaryotes and archaea. The figure was generated using the ENDscript server [77].

slowly than EcRecA. Structurally, these differences increase the compressed pitch of the helical filament, reorient the CTD and increase the positive electrostatic potential of the inner surface along the central axis of the filament, which is likely to modify interactions with DNA. However, the variations between DrRecA and EcRecA do not appear to be related to different monomer–monomer interactions or structures. Additionally, unique amino acids are found in the DrRecA structure, e.g. Gly82 corresponds to Ser70 in EcRecA, which is implicated in the interaction with the nucleotide cofactor and nearly invariant across numerous bacterial sequences [21]. Finally, DrRecA has an enhanced DNA binding affinity (dsDNA and ssDNA) [22].

Formation of the presynaptic filament

The first step of homologous recombination is the binding of RecA to ssDNA in a sequence non-specific manner. The nucleoprotein filament thus formed is called the presynaptic filament. In this first conformation, in which the protein is inactive because of the absence of its cofactors, RecA interacts with five nucleotides [23,24].

Binding of RecA to the ssDNA is realized through positive residues (Arg169, Arg176, Arg196, Lys198, Lys216) that are located in the core domain of the protein and interact with the phosphate backbone of DNA [13]. These residues belong to the ssDNA binding site of RecA. Interactions between site I and ssDNA results in the binding of ssDNA to the inner surface of RecA (Figure 7).

Residue Asp161 of loop L1 guarantees the specificity of site I for ssDNA through the creation of a steric barrier and electrostatic repulsion to the complementary strand that prevent dsDNA binding [25]. Thus, by using the negative charge of Asp161, loop L1 discriminates the ssDNA from the dsDNA in the presynaptic filament formation step. As a consequence, *in vitro*, the interaction between bare RecA monomers and dsDNA prior to ssDNA binding results in the formation of a dead-end complex unable to find homology. *In vitro*, the mutation D161A reduces the quantity of these products by suppressing the electrostatic repulsion and allowing dsDNA to bind to the primary site reversibly. *In vivo*, ssDNA specificity appears essential to the DNA repair system in which RecA binds single-stranded tracts of DNA; nevertheless, the D161A mutant has a *recA*$^+$

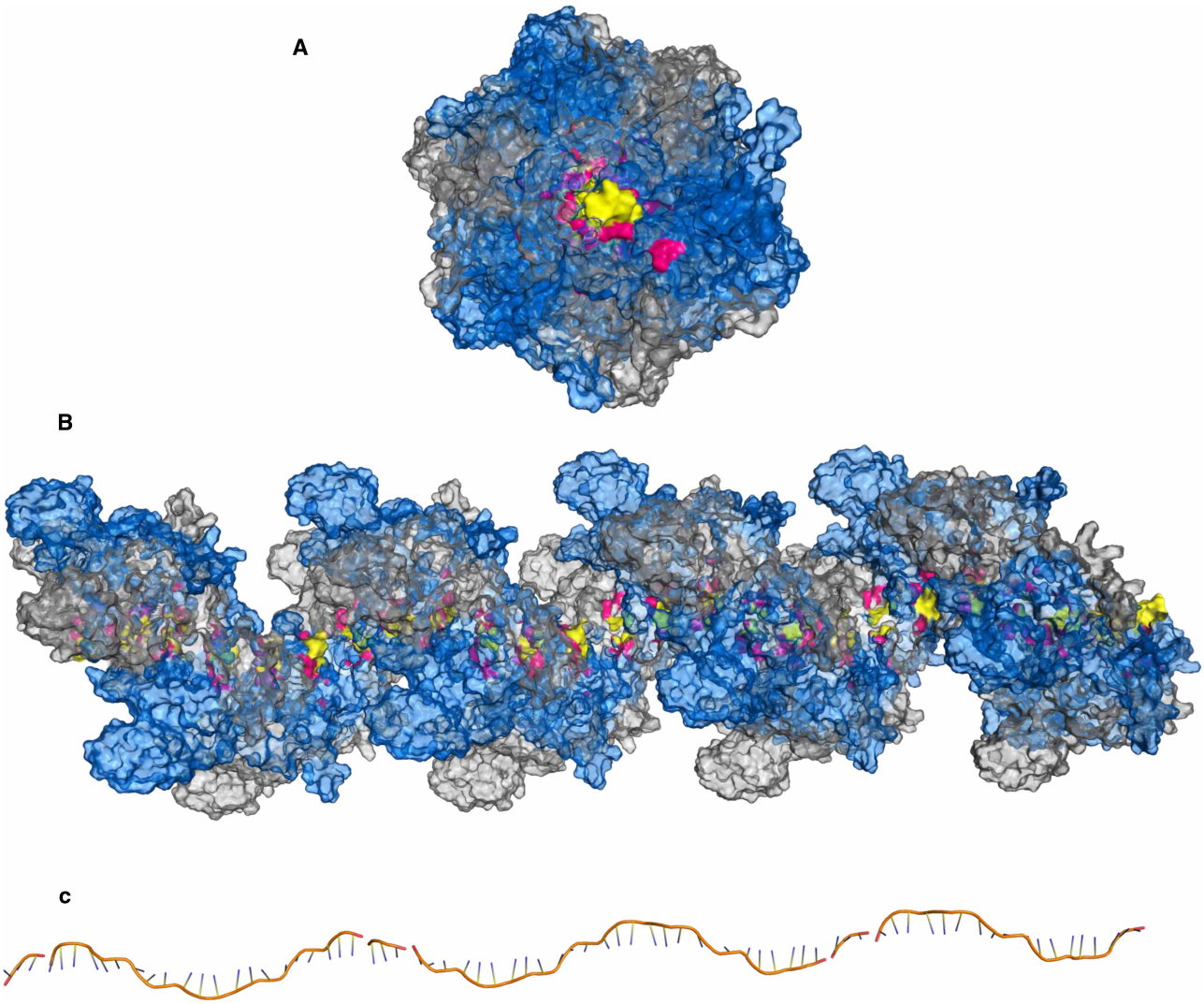

Figure 7. 3D structure of the RecA–ssDNA nucleoprotein filament.

ssDNA binds to the inner surface of RecA along the central axis, interacting with the positive residues Arg169, Arg176, Lys198 and Lys216, which belong to site I of the protein (residues 157–216). Once the nucleoprotein filament is active, it adopts a right-hand helical shape with six monomers per turn, and the ssDNA is organized into near B-form base triplets separated by 7.1 Å. (**A,B**) End and side views, respectively, of the 3D structure of the RecA–ssDNA nucleoprotein filament. The RecA monomers are coloured successively in grey and blue; the residues Arg169, Arg176, Lys198 and Lys216, in bright pink; and the ssDNA, in yellow. (**C**) Structure of the ssDNA inside the RecA filament. The figure was generated using the PyMol program [78] and models provided by Prentiss and Prevost [54].

phenotype. This is because, *in vivo*, RecA is generally bound to ssDNA by recombination mediators (RecFOR or RecBCD), so the specificity is not essential for recombination.

To cover the ssDNA in its entirety, ssDNA binding is realized in two steps: a nucleation step during which RecA monomers simultaneously bind to the ssDNA at different points, spontaneously or with the help of RecA loading machinery (RecBCD or RecFOR); and an extension step during which a contiguous filament of RecA monomers along the ssDNA is formed by the addition of RecA monomers. The nucleation step requires a dimer of RecA and is a slow and stochastic kinetic process. The extension is rapid and bidirectional, albeit faster in the $5' \rightarrow 3'$ direction than in the opposite direction [26].

SSB–ssDNA–RecA interactions are essential during the formation of the presynaptic filament. SSB can inhibit the formation of the filament or stimulate it, depending on the cellular requirements for DNA repair. In

Biochemical Society Transactions (2019) 47 1511–1531
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fact, one tetramer of SSB wraps 35 or 65 nucleotides around itself, depending on the SSB binding mode (which in turn depends on salts, the tension on ssDNA substrates and free SSB concentrations); this wrapping protects the ssDNA from being degraded and stabilizes it. In addition, SSB denatures the local ssDNA secondary structure where RecA could not bind [27]. SSB and RecA compete for ssDNA substrates. Depending on the cellular environment, RecA can displace SSB and bind to the ssDNA with an improved linearity in the nucleoprotein filament. This displacement is enabled by RecA–ssDNA interactions that compete with SSB–ssDNA interactions using accessory proteins (RecFOR proteins) but also by RecA–SSB direct interactions. This last pathway is kinetically slow and promotes an unstable interaction between RecA and SSB [28].

Some RecA variants with altered SSB displacement activity and a different behaviour towards secondary structure highlight the importance of the whole organized structure of the protein in ssDNA binding. For example, RecA proteins with 6, 13 and 17 C-terminal amino acid deletions are increasingly able to displace SSB from ssDNA and bind to secondary structural elements [29,30]. It has been proposed that the negatively charged parts of the C-terminus participate in a network of protein surface salt bridges; their disruption could consequently increase the rate of RecA association with DNA. There are also point mutations that increase the SSB displacement and binding to secondary structures, such as E38K (RecA730) and V37M (RecA803). These mutations can also promote the disruption of the same extensive salt bridge network. It is important to note that these two properties (SSB displacement and binding to secondary structures) are not always coupled: RecA441 (G38K + I298V) is proficient at displacing SSB but binds DNA with secondary structure as well as wild-type RecA does. *In vivo*, these three mutants have FRE values 2- to 8-fold greater than those of the wild-type protein [31,32].

Activation of the presynaptic filament

The presynaptic filament is first in an inactive conformation called state O. The filament then interacts with its cofactors, a nucleotide triphosphate (usually ATP but also dATP) and Mg^{2+}, to change into an active conformation called state A [6]. In this state, a stretched conformation is maintained, and the helical pitch of the filament is increased from 65 to 95 Å [33]. Then, each RecA monomer that interacts with five nucleotides in the inactive and compressed conformation will interact with only three nucleotides in the active conformation.

In this way, the resulting nucleoprotein filament has a right-handed helical shape with six monomers per turn. The ssDNA is situated along the central axis of the protein on the inner surface, where it adopts a particular organization into near B-form base triplets separated by 7.1 Å between adjacent triplets (Figure 7). In this configuration, ssDNA is extended 1.5 times further than its normal B-form configuration, with a non-uniform extension [34]. This ability of RecA to extend the DNA is known as stretching. The stretching is essential for the homology search and the strand exchange. Thus, mutant RecA V225I (RecA142), which is able to bind the ssDNA but not stretch it, cannot catalyze the DNA strand exchange [35].

In the presence of ADP, the complex re-adopts an inactive conformation. *In vitro*, non-hydrolysable ATP analogues such as ATPγS or ADP•AlF^{4-} can also be used to maintain active presynaptic filaments. dATP is more effective than ATP in this process [24,36].

Mg^{2+} is required for ATP hydrolysis but also interacts with the C-terminal tail of the protein [14,37]. The role of this latter interaction will be described in the section 'Homology search'.

ATP binding and hydrolysis

The nucleotide-binding site is localized in the interface between RecA monomers, making the formation of an active complex a cooperative process that is accelerated when the number of RecA monomers bound to ssDNA increases. Specifically, the ATP binding site where the phosphate groups of the ATP bind is a loop with the sequence 66-GPESSGKT-73, which matches the consensus Walker A-box (or P-loop) sequence (G/A)XXXXGK(T/S) found in numerous NTP-binding proteins [38]. Additionally, Gln194 is thought to be a mediator of the allosteric effect of ATP, serving as a link between the phosphate and loop 2, which is implicated in ssDNA binding site I function (Figure 8) [39].

A [KR] × [KR] motif catalyzes ATP hydrolysis and co-ordinates the ATP hydrolysis cycles between subunits or domains in numerous enzymes such as helicases and recombinases. In RecA, this motif, composed of the residues Lys248 and Lys250, is present at the subunit–subunit interface. Additionally, these amino acids positively co-operate across the interface with the Glu96 residue of the neighbouring monomer. In fact, Lys248 or Lys250 and Glu96 are located at the subunit–subunit interface near the nucleotide-binding site, close and opposed across the interface (Figure 9). Therefore, they communicate conformation changes induced by ATP

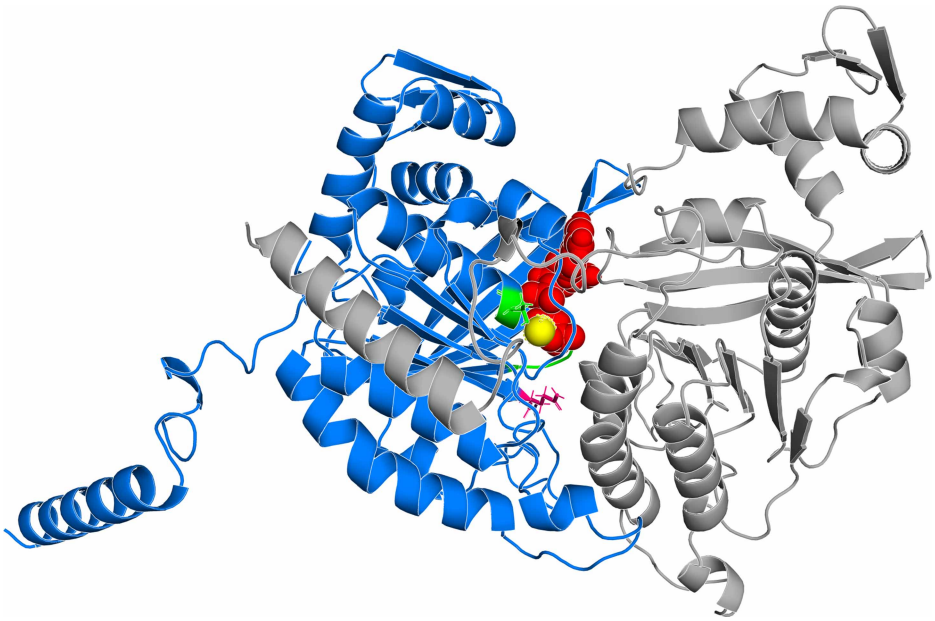

Figure 8. 3D structure of an ATP molecule bound between two RecA monomers.

ATP binds to the ATP binding site 66-GPESSGKT-73, which is localized in the interface between RecA monomers. Additionally, Gln194 is thought to be a mediator of the allosteric effects of ATP. ATP is represented by red spheres, the Mg ion is a yellow sphere, the ATP binding site is in green, and the Gln194 is in bright pink. The RecA monomer that binds the ATP molecule is shown in blue, and the adjacent RecA monomer is shown in grey. The figure was generated using the PyMol program [78] and models provided by Prentiss and Prevost [54].

hydrolysis to the adjacent subunits of the nucleoprotein filament. This relation is supported by the absence of or reduction in ATPase activity in the mutants RecAK248R, RecAK250R and RecAE96D. Furthermore, a mixture of the simple mutants RecAK248R and RecAE96D forms a nucleoprotein filament with four possible subunit–subunit interfaces (the wild-type interface K248/E96 and the mutant interfaces R248/E96, K248/D96 and R248/D96) and results in partially restored ATP activity. The same behaviour is observed for the mixture of mutants RecAK250R and RecAE96D [40,41].

Mutation in the nucleotide-binding site can also alter the hydrolysis activity without altering the binding affinity, as observed with the mutants RecAK72R, which binds but does not hydrolyze ATP [42], or RecAS69G, which has different hydrolysis rates than the wild-type protein [38].

In addition to the role of ATP binding in the activation of the presynaptic filament, ATP hydrolysis influences RecA activities in different ways.

First, during presynaptic filament formation, RecA-ATP has a higher affinity than RecA-ADP for DNA; thus, ATPase activity results in a dynamic cycle of binding and dissociation. As monomer–monomer interactions stabilize the nucleoprotein filaments, dissociation of RecA-ADP monomers occurs predominantly from filament ends [43]. This activity limits the length of the filaments [44]. Single-molecule fluorescent resonance energy transfer (FRET) assays showed that cooperativity between neighbouring monomers provides a time window for the exchange of ADP for new ATP, keeping the filament in an active and stretched conformation among the continuous cycles of ATP hydrolysis in an ATP-rich environment [45]. In this environment, the ATP/ADP molar ratio is greater than 2–3 [46].

Second, ATP hydrolysis participates in the nucleation and extension steps. The nucleoprotein filament can be formed by multiple patches of RecA filaments on the same ssDNA that need to merge with each other to form a contiguous filament. As each RecA monomer occupies three bases of DNA, there may remain one or two nucleotide gaps between adjacent patches. It has been demonstrated by a single-molecule fluorescence assay that ATP hydrolysis drives a reorganization to eliminate these gaps via allosteric changes and to improve the filament integrity. This process is influenced by the χ sequence (recombination hotspot, 5′-GCTGGTGG-3′, increasing the frequency of genetic exchange in its vicinity) [47], which is recognized by the RecBCD loading

Biochemical Society Transactions (2019) **47** 1511–1531
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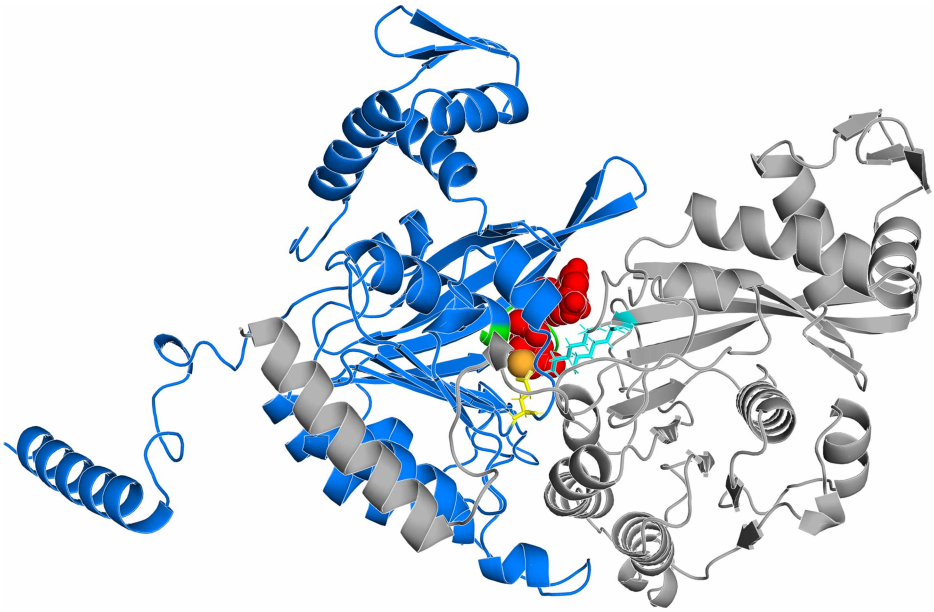

Figure 9. 3D structure of two RecA monomers and the residues implicated in ATP hydrolysis.
The observed ATPase activity implicates the presence of the [KR] × [KR] motif containing the residues Lys248 and Lys250 at the subunit–subunit interface. Additionally, these amino acids positively co-operate across the interface by interacting with the Glu96 residue of the neighbouring monomer. These residues are located at the subunit–subunit interface near the nucleotide-binding site, close and opposed to each other across the interface. The RecA monomers are represented in blue and grey. The ATP molecule (in red spheres, with an Mg molecule in orange) binds at the ATP binding site of the blue monomer (represented in green) at the interface of the two monomers. ATP hydrolysis is catalyzed by the residue Glu96 of the blue monomer, in yellow, and by the residues Lys248 and Lys250 of the grey monomer, in cyan. The figure was generated using the PyMol program [78] and using the models provided by Prentiss and Prevost [54].

machinery, that recognizes more generally the TGG sequence: RecBCD loads RecA onto the ssDNA and aligns the first patch to the χ sequence, while ATP hydrolysis changes the phase of the second patch to merge with the first one. Multiple rounds of loading, extension and joining are needed to rapidly generate a long and homogeneous nucleoprotein filament (Figure 10) [48].

ATP hydrolysis also accelerates the homology search and is essential to promote extensive DNA strand exchange. These two features are discussed below in the respective sections, 'Homology search' and 'Formation of the postsynaptic filament and strand exchange'.

Once the presynaptic filament is activated by the two cofactors ATP and Mg^{2+}, the homology search can begin.

Homology search

The nucleoprotein filament composed of an ssDNA strand covered by RecA monomers performs a genome-wide search to find a homologous dsDNA sequence.

First, the filament interacts non-specifically with dsDNA through positively charged residues of the NTD that form a helical patch along the outer surface of the nucleoprotein filament: Lys6, Lys8, Lys19 and Lys23. These residues contact the phosphate groups of dsDNA by electrostatic interactions [49]. Subsequently, dsDNA can then interact with the positive residues of the CTD (Lys280, Lys282, Lys286 and Lys302) through the formation of salt bridges [50]. This region of the protein represents a gateway to the dsDNA binding site, also called site II. Site II is composed of residues 226–245, is proximal to the ssDNA bound to site I within the interior of the filament groove, and has a weaker affinity for DNA than site I. dsDNA interacts notably with the positive residues Arg226, Arg227, Arg243 and Lys245 (Figure 11) [13]. All these successive interactions between the different RecA domains and the dsDNA implicate large structural movements between the NTD and the CTD.

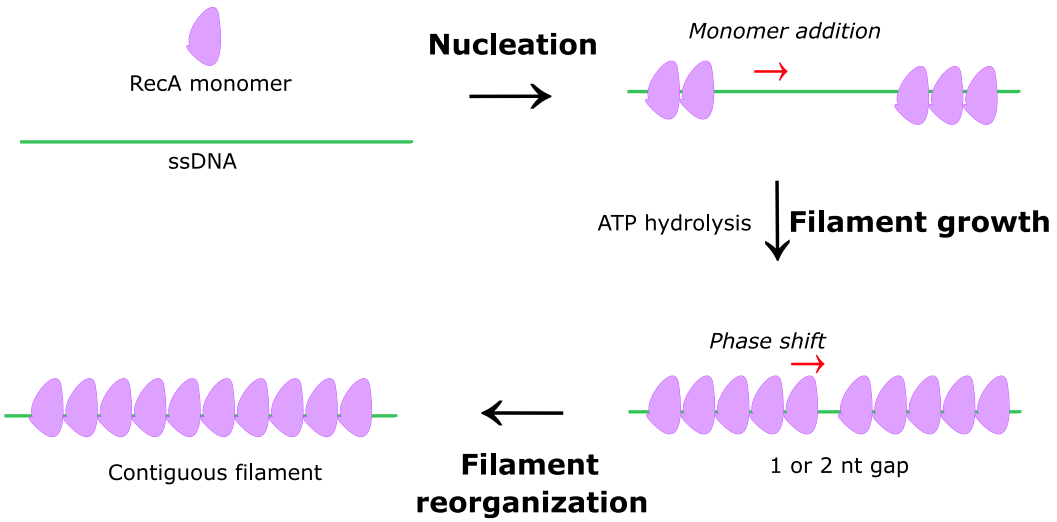

Figure 10. Filament reorganization during the formation of the presynaptic filament.

RecA monomers (in violet) nucleate on ssDNA (in green). This phase is followed by the extension of the filament by monomer addition, mainly in the 5′ → 3′ direction. As one monomer interacts with three nucleotides and many patches of RecA polymers are formed at a time, one or two nucleotide gaps can remain between patches. The patches can reorganize to suppress these gaps and form a contiguous filament due to the RecA ATP hydrolysis activity.

The C-terminal tail also regulates dsDNA binding. In fact, deletions in the C-terminal tail, particularly between positions 339 and 346, enhance binding to dsDNA. It has been proposed that the negatively charged tail regulates dsDNA binding by electrostatically repelling the phosphate group of the DNA [50] and that it interacts with the NTD and the dsDNA gateway of the CTD participating in the non-specific binding of dsDNA [29].

The binding of at least two Mg^{2+} molecules to the C-terminal tail eliminates inhibition by increasing the protein flexibility, activating the nucleoprotein filament and allowing the access of the dsDNA to its binding site. Thus, the deletion of the C-terminal tail renders RecA insensitive to Mg^{2+} with similar activity as full-length RecA in the presence of saturating amounts of Mg^{2+} [37]. *In vivo*, the deletion increases the FRE: the FRE of *E. coli* RecA truncated by 17 amino acids is enhanced by 4-fold [51]. It has not been established whether the tail has a regulatory function or just stabilizes RecA in the cell, in which case, the negative effect would be just a byproduct. Mutants of RecA with 13–17 residues deleted from the C-terminus are not more UV-sensitive or ionizing radiation-sensitive than the wild-type protein, but their sensitivity to the DNA cross-linker mitomycin C is significantly increased. This suggests a possible role of the C-tail directly at the sites of interstrand cross-link repair or with other proteins involved in the repair process [29]. *In vitro*, the Mg^{2+} effect is used to alter and regulate the performance of RecA by modifying the chemical environment: this effect is greater at 10 mM Mg^{2+} than at the 1–2 mM physiological cellular concentration of free Mg^{2+}[52]. However, this cellular concentration is hotly debated and can range from 3 to 18 mM. Likewise, the addition of volume-occupying agents such as polyethylene glycol or polyvinyl alcohol has the same effect [30]. Mg^{2+} and the other agents may stabilize a conformation of the C-terminus that does not inhibit the protein activity, exposing the RecA binding site in such a way that RecA is increasingly efficient, in an increasingly open and hence active conformation. The pH also affects in this phenomenon: as pH decreases, the binding is accelerated and the dissociation of the monomers from the nucleotides is reduced [53]. In fact, the assembly of RecA on SSB–ssDNA is strongly stimulated by reducing the physiological pH of 7.5–6.5 [26]. This justifies the importance of the *in vivo* cellular mediator proteins quoted previously. Nevertheless, the binding dependence on the potassium concentration is low.

Once bound to site II, dsDNA acquires a particular conformation with Watson–Crick base-paired triplets with a rise of 8.4 Å between adjacent triplets [34] and a bent junction between B-form dsDNA not bound to RecA and dsDNA in site II (Figure 11). This conformation locally destabilizes the double strand and leads to base-flipping; hence, the bases of the incoming strand of the dsDNA can pair transiently with the bases of the

Biochemical Society Transactions (2019) **47** 1511–1531
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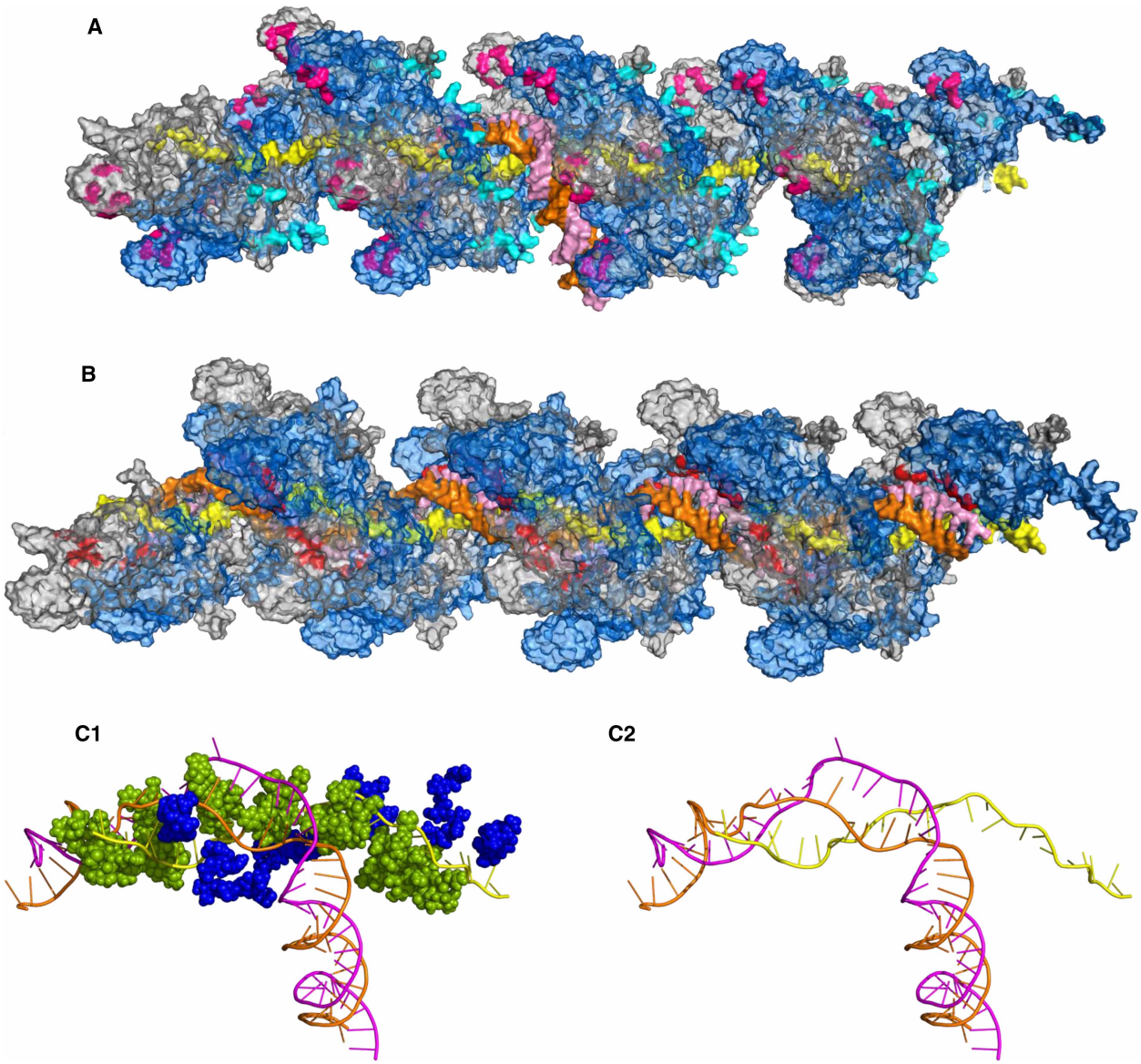

Figure 11. 3D structure of the RecA–ssDNA–dsDNA nucleoprotein filament.

(**A**) First, the filament interacts non-specifically with dsDNA through the positive residues of the NDT that form a helical patch along the outer surface of the nucleoprotein filament: Lys6, Lys8, Lys19 and Lys23, represented in bright pink. Then, dsDNA interacts with the positive residues of the CTD, i.e. Lys280, Lys282, Lys286 and Lys302 (represented in cyan), to finally bind to site II. In the 3D structure, an ssDNA in site I at the inner surface of the filament is shown in yellow, and a dsDNA in site II is shown in orange and pink. The dsDNA has a particular conformation of Watson–Crick base-paired triplets with a rise of 8.4 Å between adjacent triplets and a bent junction between B-form dsDNA not bound to RecA and dsDNA in site II. (**B**) dsDNA (shown in orange and pink) binds site II, interacting notably with the positive residues Arg226, Arg227, Arg243 and Lys245, shown in red. This site is proximal to the ssDNA bound to site I within the interior of the filament groove, represented in yellow. This structure is stable for short regions of only less than 12 base pairs, but it is presented to aid visualization of the different sites of the protein. (**C1, C2**) The homology search starts with the flipping of base duplets of the dsDNA (shown in orange and pink) that are paired with the bases of the ssDNA (in yellow) to check for homology. The steric hindrance of loops L1 (in blue) and L2 (in green) necessary for this process is shown in figure C1. The figure was generated using the PyMol program [78] and models provided by Prentiss and Prevost [54].

ssDNA by Watson–Crick interactions and thus iteratively test the homology between the two strands. Paradoxically, this testing stage is rapid and stringent, requiring correct and strong interactions between the partners and simultaneous rapid reversal of incorrect interactions. Based on these considerations, a model has

been developed by docking and molecular dynamics simulations. It appears that the tension resulting from the dsDNA extension naturally divides the search into stages. After the non-specific binding of dsDNA to site II, the phosphates from the displaced strand triplets strongly interact with the basic residues Arg226, Arg227, Arg243 and Lys245, contributing to the uncoiling of dsDNA. The hydrophobic residues of the L2 loop (Met202 and Phe203) intercalate between triplets, creating a tense conformation and destabilization of the internal pairing and stacking interactions. Thus, the two first bases of each triplet flip spontaneously and iteratively in the $5' \rightarrow 3'$ direction (Figure 12). The next duplet flips only when the first is stably bound. The third base of each triplet cannot flip due to steric hindrance created by loops L1 and L2 (with residues Met164 and Ile199, respectively) and interacts with the bases of the outgoing strand (Figure 11). Flipping is also favoured by the hydrophobic environment in which duplets are confined [13,54].

In the case where correct base pairing is above a threshold of eight bases, the nucleoprotein complex with the triple helix is stabilized by the relocation of the L2 loop: when three successive duplets are flipped, a conformation change occurs by loop L2 displacement; the two non-flipped bases also flip, reducing mechanical stress and increasing base pair stability (Figure 12). In fact, single-molecule experiments revealed two distinct time regimes during which the ssDNA nucleoprotein filament interrogates the dsDNA for homology by multiple binding and release steps. During the first time regime, fragments with fewer than eight base pairs of homology are rapidly sampled and rejected within a few seconds. Then, fragments with eight or more base pairs of homology acquire a metastable conformation and present exponentially increasing lifetimes with three

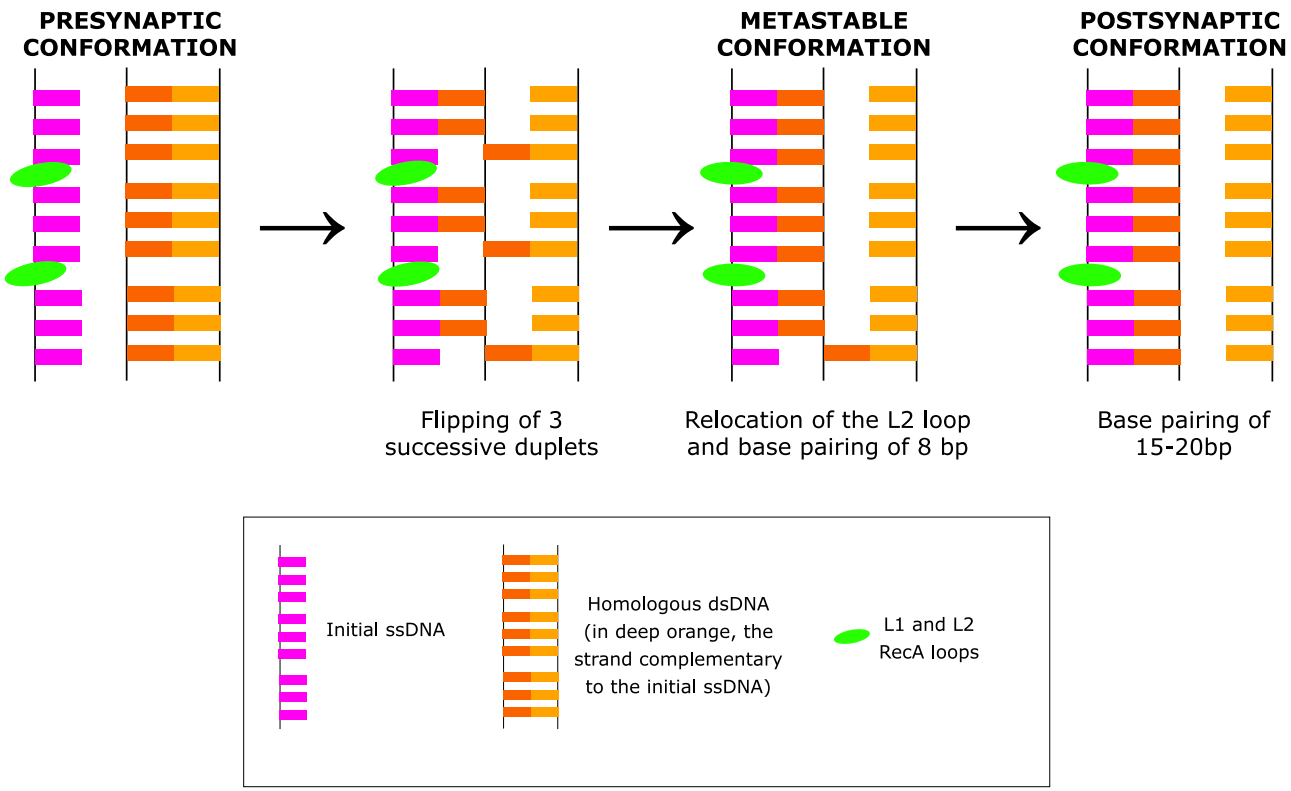

Figure 12. Mechanism of acquisition of the RecA postsynaptic conformation.

In the presynaptic conformation, the dsDNA bound to site II acquires a conformation with B-form triplets. During the homology search, the first two nucleotides of each triplet flip successively as duplets. The next duplet flips when the previous one is stably bound (i.e. when the homology is correct). The third base does not flip because of the steric hindrance created by loops L1 and L2. When three successive duplets are flipped, a conformation change occurs by L2 displacement, and the two unflipped bases also flip, reducing mechanical stress and increasing base pair stability. When this threshold of eight bases is exceeded, the nucleoprotein complex acquires a metastable conformation. Finally, the next triplets flip successively until 15–20 base pairs are interacting with the initial ssDNA. The filament is thus in the postsynaptic conformation: the interaction between dsDNA and ssDNA is stabilized, and RecA can proceed to exchange strands.

Biochemical Society Transactions (2019) 47 1511–1531
https://doi.org/10.1042/BST20190558

base pair increments, each step coinciding with an energetic signature of 0.3 k_BT [55]. An asymptotic binding value is approached when 15–20 contiguous homologous base pairs are in a strand exchange conformation [56]. The base pairing of the flipped duplet of the first stage is much less stable than the base pairing of a complete triplet, explaining why the first stage homology test progression is faster than the second. Therefore, during recombination, the RecA role is to establish a particular DNA structure that then governs the mechanism, energetics and fidelity during recombination, making this structure the most energetically favourable for strand exchange.

DNA curtain technology for imaging protein–nucleic interactions at the single-molecule level showed that during the first step, RecA requires perfect Watson–Crick interactions to stabilize pairing interactions. Then, RecA can step over base triplets harbouring a single mismatch but do not stabilize them; in contrast, single nucleotide insertions disrupt base triplet recognition [57].

ATP hydrolysis plays a role in the homology search by accelerating the micro-homology testing up to 4-fold. In fact, it has an impact on the turnover of dsDNA fragments bound to the RecA presynaptic complex through 8–15 base pair tracts of micro-homology. This enhancement is supposed to be triggered by ATP-dependent conformational changes within the presynaptic filament that enable the release of the complementary dsDNA strand [56].

Numerous investigations have been undertaken to explain how the presynaptic filament can realize the homology search in a metabolic timeframe, which is accomplished by a combination of different processes. Contrary to what was first thought, ATP hydrolysis is dispensable in the homology search: RecA proceeds by a biomolecular collisional process rather than by an ATP-dependent directed motion. Both experimental studies and mathematical modelling approaches have shown that efficient searching occurs when the target dsDNA is coiled, allowing multiple simultaneous contacts of the nucleoprotein filament with different sites within the same dsDNA. This process is called intersegment sampling [58,59]. If the DNA is supercoiled, the process is increasingly efficient as the local density of dsDNA increases. Furthermore, the flexibility of the CTD allows DNA sliding and the testing of the DNA in multiple registers (i.e. a different register takes place when the DNA is shifted one base) without free diffusion: four different registers can be tested without changing the binding of the dsDNA to the CTD, and the binding can also be transferred to the neighbouring CTDs [54]. Finally, static and dynamic atomic force microscopy experiments have shown the importance of cooperative RecA clustering: multiple filaments interact on the same dsDNA simultaneously to ensure a high interaction rate and increasingly rapid and efficient interactions through parallel searches and confinement of reactive species. This is followed by a slower phase where the filaments successfully located at a site of sequence homology remain bound and progress to strand exchange while the others dissociate from the dsDNA, resulting in a reduction in the interaction activity of the RecA filaments [60]. Therefore, the combination of all these mechanisms offers an explanation of the genome-wide homologous search on a relevant metabolic timescale.

Formation of the postsynaptic filament and strand exchange

When 15–20 contiguous homologous base pairs are in a strand exchange conformation, the interaction between dsDNA and ssDNA is stabilized; the RecA protein thus proceeds to exchange strands by propagating the unrolling of dsDNA triplets, binding to the ssDNA via Watson–Crick interactions and successively displacing L2 loops, producing a heteroduplex DNA composed of the ssDNA and one strand of the dsDNA. The postsynaptic complex is favoured because site II has a higher affinity for the displaced ssDNA, a product of the DNA strand exchange, than for the dsDNA, which is the reaction substrate [61]. The 33% decrease in the rate of ATP hydrolysis associated with the DNA pairing and strand exchange reaction reflects a new change in the state and conformation of the nucleoprotein filament [62]. In fact, when DNA pairing occurs, the filament changes its conformation from an A-state to a P-state [6]. It can then initiate and propagate strand exchange.

Homologous pairing and short strand exchanges require binding of ATP but not its hydrolysis [42,63]. Extensive DNA strand exchange (with strands longer than 3 kbp) is generally coupled to ATP hydrolysis, allowing filaments to function as rotary motors [64]. In the presence of ATP hydrolysis, the reaction is robust and unidirectional ($5' \rightarrow 3'$) and bypasses short heterologous sequences and topological barriers [57,65]. In this context, it is important to note that optimized pairing can have a negative effect on extensive DNA strand exchange, especially in the absence of ATP hydrolysis, because strong pairing can lead to nonproductive DNA–DNA interactions and thus topological barriers. The optimal filament DNA affinity is reached by a compromise

between the filament being trapped and not reaching the DNA at all [59,66]. Thus, the mutant RecAK72R, which binds ATP without hydrolyzing it, presents efficient DNA pairing in the presence of high levels of Mg^{2+} but does not progress to extensive strand exchange, while low levels of Mg^{2+} allow for a slow completion of the reaction [67]. Additionally, ATP hydrolysis promotes the disassembly of RecA–DNA filaments after completion of strand exchange [68]. Furthermore, nucleoid surface spreading, immunostaining and super-resolution microscopy studies performed on ATPase-defective mutants RecAK250N and RecAE96D *in vivo* revealed that ATP hydrolysis also promotes disassembly from undamaged regions of dsDNA, preventing the accumulation of dead-end and potentially toxic complexes [69].

SSB facilitates DNA strand exchange by binding to the displaced single strand of duplex DNA and facilitating its release from the filament [5].

The process of homologous recombination in relation to the RecA structure is illustrated in Figure 13.

Homologous recombination as a cooperative process

Homologous recombination, from the generation of the presynaptic filament to formation of the postsynaptic filament and heteroduplex DNA, is a cooperative process: all the RecA monomers bound to the ssDNA participate together in the process. Therefore, the monomer–monomer interactions and the flexibility of the protein that assures proper folding are crucial for its activity.

Mutations in specific protein regions that affect the flexibility of the protein (Figure 4) have important consequences for its recombinogenic activity. For example, the L29M mutation increases the flexibility of the RecA polymer, leading to an increase in the recombinogenic activity that is caused by reduced dissociation of the presynaptic complex, increasingly active displacement of SSB from ssDNA and increased interactions with

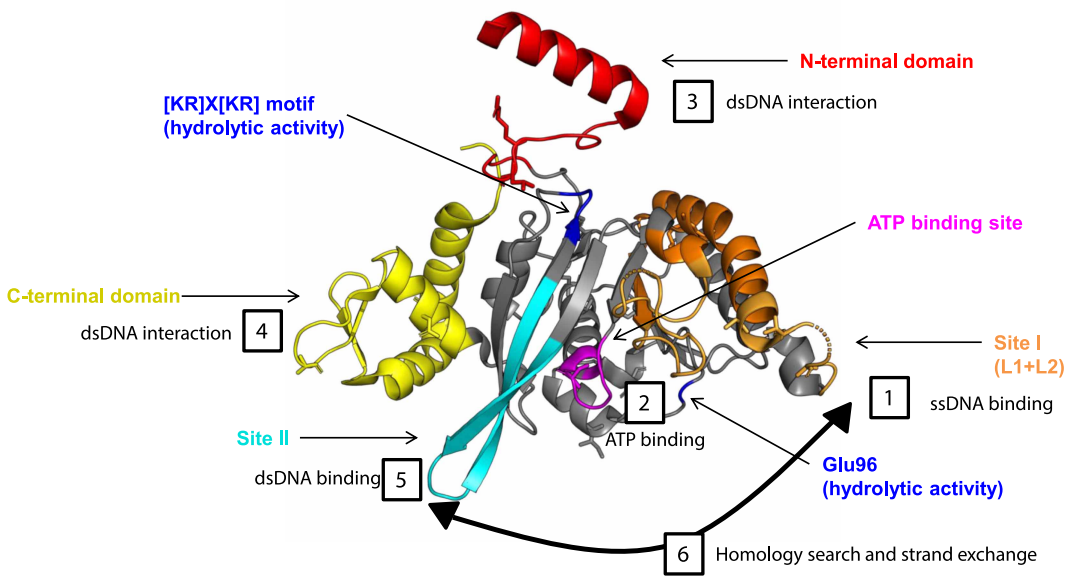

Figure 13. RecA 3D structure and its relationship with the activities involved in recombination.

The different domains of the protein are coloured: in red, the NTD; in pink, the ATP binding site; in blue, the hydrolytic residue Glu96 and the hydrolytic motif [KR] × [KR]; in orange, site I that includes loops L1 and L2; in cyan, site II; and in yellow, the dsDNA gateway in the CTD. The numbered boxes and the accompanying text indicate the different steps of recombination: first, the single-stranded DNA (ssDNA) binds to site I of the protein, forming a nucleoprotein filament (1). Then, ATP, a RecA cofactor, binds to the ATP binding site and activates the filament (2). After that, RecA performs a homology search to find a homologous double-strand (dsDNA). In this process, dsDNA interacts first with the NTD (3) and then with the CTD (4), by which it can move to site II, where it binds (5). If the bound dsDNA is homologous to the ssDNA, strand exchange is performed; if it is not, the dsDNA is released (6). During these steps, the nucleoprotein filament has hydrolytic activity, and this hydrolysis is carried out by the [KR] × [KR] hydrolysis motif containing Lys248 and Lys250, which co-operate with Glu96 on the other monomer. The figure was generated using the PyMol program [78].

Biochemical Society Transactions (2019) **47** 1511–1531
https://doi.org/10.1042/BST20190558

dsDNA [16,18]. Additionally, in the CTD, the two amino acids D276 and K302, which form a salt bridge, are implicated in the conformation shifts and protein flexibility. Hence, the mutations D276A and D276N alter the protein flexibility by eliminating the salt bridge and confer hyper-recombination activity to RecA. In fact, the suppression of the salt bridge would increase the freedom of the CTD to interact with dsDNA and initiate DNA pairing and/or would render the mutant less able to shift from an active conformation to an inactive one. These two mutants have been discovered by directed evolution experiments based on ionizing radiation treatment of *E. coli* and selection of colonies displaying an enhanced recombination repair system. Specifically, the RecA D276A and RecA D276N proteins create more and smaller nucleoprotein filaments than the wild-type protein; these filaments promote strand exchange more efficiently and are less inhibited by ADP. Another mutation, A289S, has been discovered by the same technique, but the origin of the recombination improvement has not been studied [70,71]. These mutations are described in Table 1

Furthermore, the loops L1 and L2 have major roles in the protein activity with their participation in the homology search due to their flexibility. Thus, mutations in these loops (for example, substitution at positions R196, F203 or E207 and substitution of K198 by an amino acid other than R) have deleterious effects on recombination, from its reduction to its total inactivation [72,73].

The other crucial aspect of the cooperation between monomers is the monomer–monomer interface itself. Mutations in the two regions that constitute the interface (described in the 'Recombinases and structural organization' section) alter the recombinogenic activity, highlighting the importance of subunit interactions for all the genetic and biochemical activities of the RecA filament. Thus, the mutations K6A, G136N + V142I and I228T decrease the recombinogenic activity, while other mutations such as R28A, R28D, I102D, I159M + S162A + M164V, D112R or R28D + D112R lead to an increase [16,51]. These mutants have been found by mutagenesis experiments targeting the monomer–monomer interface and characterized by FRE measurements. They present variations in the different RecA activities, supporting the fact that homologous recombination is a cooperative process and that a change in the monomer–monomer interface has an effect on all the enzyme activities. Thus, R28A and R28D improve ssDNA binding and D112R boosts DNA pairing, while K6A reduces affinity for ssDNA. The hyper-recombination mutations are described in Table 1. Therefore, the modification of the subunit–subunit interface to alter the recombinogenic activity has been exploited regularly. For example, random mutagenesis at the interface between residues 79 and 137 followed by directed evolution to enhance the capacity for conjugational recombination has provided different RecA mutants with an enhanced capacity, such as RecAI102L, RecAV79L and RecAE86G/C90G [74]. These mutants catalyze the pairing of homologous DNAs more efficiently than the wild type.

Hyper-recombinant forms of RecA

RecA possesses an inherently broad functional range that is not optimized for the recombination function alone. Thus, to retrieve the high potential of RecA for recombination, modification of modulatory proteins and of the chemical environment but mostly the suppression of the C-tail and point mutations are exploited *in vivo* (in the context of homologous recombination of truncated sequences, conjugational recombination, and resistance to UV, mitomycin C and ionizing radiation) and *in vitro* (in strand exchange assays). In fact, small changes in the amino acid sequences may uncover a high level of recombination potential. Additionally, chimeric proteins combining EcRecA and PaRecA have been produced, e.g. RecAX45 has one interface from EcRecA and the other from PaRecA, while RecAX53 has 12 amino acids from PaRecA in the middle of the EcRecA structure; both chimeras enhance the FRE up to 9-fold [16,32].

There are limits to the levels of hyper-recombination tolerated *in vivo*, which explains why the frequency of recombination can be enhanced no more than 50-fold in experiments [51] and why the phenotype of hyper-recombination mutants as RecA D112R is lost over the course of 70 cell generations. In fact, mutations create selective pressure because of the induction of barriers for other processes in DNA metabolism and physical DNA availability, which results in a barrier to cell reproduction [9].

The main described mutations named in this text that result in a RecA hyper-activity are described in Table 1.

In conclusion, RecA activity can be optimized by modification of the modulatory proteins and the chemical environment but mostly by the suppression of the regulatory C-tail and by point mutations. Additionally, there are recombinases from other species that have enhanced recombination activity (e.g. those from *P. aeruginosa* and *D. radiodurans*). A combination of the point mutations summarized here could be additive and improve

Table 1 Hyper-recombination RecA point mutations

Mutation [References]	Modification	Biochemical effect	Experimental discovery	Experimental study
R28A or R28D [15,16,51]	Monomer–monomer interface modification	Increased ssDNA affinity	Mutagenesis at the interface	*In vivo* FRE measurements *In vitro* characterization (filament study by electron microscopy, ATP hydrolysis assay, DNA binding assay by gel shift, strand exchange assay)
L29M [16,18]	Monomer–monomer interface and flexibility modification	Increased dsDNA affinity, filament dissociation, SSB displacement	Chimera PaRecA–EcRecA study	*In vivo* FRE measurements *In vitro* characterization (ATP hydrolysis assay, strand exchange assay)
E38K [30,51]	Flexibility modification	Increased ssDNA affinity, SSB displacement, binding to secondary structures	Screening of UV resistants	*In vivo* FRE measurements *In vitro* characterization (ATP hydrolysis assay, DNA pairing by FRET)
S70G [21]	ATP binding site	Coupling ATP binding — conformational changes	Study of DrRecA structure	—
V79L E86G + C90G I102L [74]	Monomer–monomer interface modification	Increased DNA binding	Random mutagenesis and directed evolution with enhanced capacity for conjugational recombination	Conjugational recombination *In vitro* characterization (ATP hydrolysis assay, strand exchange assay, D-loop formation assay)
D112R [51]	Monomer–monomer interface modification	Increased dsDNA binding and pairing, SSB displacement, binding to secondary structures	Mutagenesis at the interface	*In vivo* FRE measurements *In vitro* characterization (ATP hydrolysis assay, DNA pairing by FRET)
I159M + S162A + M164V I102D [16]	Monomer–monomer interface modification	—	Chimera PaRecA–EcRecA study	*In vivo* FRE measurements
L178I + A179T + L182I [17)	Site I	Increased ssDNA affinity	Chimera PaRecA–EcRecA study	—
D276A or D276N [70,71]	Flexibility modification	Increased number of little filaments, strand exchange, ADP inhibition	Directed evolution with enhanced ionizing radiation resistance	*In vitro* characterization (ATP hydrolysis assay, strand exchange assay, filament study by electron microscopy)
A289S [70]	—	—	Directed evolution with enhanced ionizing radiation resistance	—
ΔC17 or ΔC25 [29,30,51]	Suppression of the inhibitory C-tail	Increased dsDNA binding and pairing, SSB displacement, binding to secondary structures	Mutant studies	*In vivo* studies (UV, mitomycin C, ionizing radiation) and FRE measurements *In vitro* characterization (ATP hydrolysis assay, filament study by electron microscopy, DNA pairing assay by FRET, strand exchange assay)

Biochemical Society Transactions (2019) **47** 1511–1531
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the recombination function even more. The effect of the mutation combinations should be tested *in vivo* and *in vitro*. A mutant can have enhanced activities *in vitro* but present a deleterious effect *in vivo*. Conversely, for example, a *rec*+ phenotype *in vivo* can be explained by an enhanced stability or interaction with other components in the cell medium that are not useful in an *in vitro* environment.

Perspectives

- The study of the RecA molecular mechanisms is essential to understand homologous recombination and the way RecA performs its activity in a relevant metabolic timeframe. It is also fundamental to understand these mechanisms to engineer an effective enzyme that could be used for diagnostic applications such as recombinase polymerase amplification (RPA).

- In recent years, single-molecule experiments have highlighted several mechanisms that elucidate the protein structure/function relationship, the correlation of ATP hydrolysis with the whole recombination activity and the homology search over short timescales. The importance of protein flexibility and monomer–monomer interactions has also been underlined, as homologous recombination has been defined as a cooperative process.

- The selection of hyper-recombinogenic RecA mutants *in vivo* has limitations because of their toxicity to the balance between the different metabolic processes. Enzyme designs based on the knowledge of RecA molecular mechanisms open up promising avenues.

- Today, the RPA method is commercialized for research use in only kit configurations (TwistDx), enabling DNA or RNA amplification. This kit uses the phage recombinase UvsX in a reaction mix [75]. UvsX performs the heteroduplex extension quickly, proceeding at 12–15 base pairs per second, while RecA proceeds at 2–10 base pairs per second. Additionally, UvsX requires only one accessory protein (UvsY), while RecA requires three (RecF, RecO and RecR) [14]. However, amplification strategies centred on *E. coli* RecA that use only the recombinase, SSB and a polymerase have recently been developed [76]. The development of this field could provide alternative methods centred on new RecA mutant enzymes that perform equally as well as or better than UvsX, leading to the establishment of a competitive market.

Abbreviations

CAD, central core ATPase domain; CTD, C-terminal domain; DrRecA, *D. radiodurans* RecA; DSBR, double-strand break repair; DSBs, double-strand breaks; dsDNA, double-strand DNA; EcRecA, *E. coli* RecA; FRE, frequency of recombination exchanges; FRET, fluorescent resonance energy transfer; NTD, N-terminal domain; PaRecA, *P. aeruginosa* RecA; RPA, recombination polymerase amplification; SSB, single-stranded binding protein; ssDNA, single-strand DNA; SSGR, single-strand gap repair; WHO, World Health Organization.

Author Contribution

H.A. and S.R. proposed the subject of the review. E.V. wrote the manuscript with input from all authors.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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