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DIFFERENCES IN THE INTERACTION OF RAD51 AND UvsX RECOMBINASES WITH DNA REVEALED BY SINGLE-MOLECULE EXPERIMENTS

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Abstract. Nucleoprotein filaments formed by recombinase proteins on DNA are key structures of the homologous recombination process, which ensures the maintenance of the genome stability. Filament formation on DNA leads to a significant change in the mechanical properties of the complex, in particular to DNA lengthening, while the kinetics of filament assembly depends on mechanical tension. Using optical tweezers, this study has obtained data on the assembly dynamics of human RAD51 recombinase and bacteriophage T6 UvsX recombinase filaments on double-stranded DNA under varying mechanical tension. In the range between 3 and 12 pN, RAD51 efficiently bound to DNA, forming filaments with a high coverage. In contrast to RAD51, an efficient UvsX filament formation occurred only under high tension (12 pN), whereas UvsX binding to DNA was severely restricted at 3–6 pN. The different tendency of the two recombinases to interact with DNA reflected their adaptation to cellular environments and regulatory mechanisms governing homologous recombination.

Keywords: homologous recombination, RAD51, UvsX, RecA, cancer treatment, recombinase polymerase amplification, optical tweezers, nucleoprotein filament

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РАЗЛИЧИЯ ВО ВЗАИМОДЕЙСТВИИ РЕКОМБИНАЗ RAD51 И UvsX С ДНК, ВЫЯВЛЕННЫЕ В ЭКСПЕРИМЕНТАХ НА УРОВНЕ ОДИНОЧНЫХ МОЛЕКУЛ

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Аннотация. Нуклеопротеиновые филаменты, формируемые белками-рекомбиназами на ДНК, являются ключевыми структурами процесса гомологичной рекомбинации, обеспечивающей поддержание стабильности генома. Формирование филамента на ДНК приводит к значительному изменению механических свойств комплекса, в частности к удлинению ДНК, при этом кинетика сборки филамента зависит от механического натяжения. В работе с помощью метода оптического захвата получены данные о динамике сборки филаментов RAD51 человека и UvsX бактериофага T6 на двухцепочечной ДНК при различных силах механического натяжения. В диапазоне 3–12 пН RAD51 эффективно связывается с ДНК, формируя филаменты с высокой степенью покрытия. В отличие от RAD51, эффективное формирование филаментов UvsX происходит только при высоком натяжении (12 пН), тогда как при значениях 3–6 пН связывание UvsX с ДНК существенно ограничено. Различная склонность двух рекомбиназ к взаимодействию с ДНК отражает адаптацию к клеточным условиям и механизмам регуляции гомологичной рекомбинации.

Ключевые слова: гомологичная рекомбинация, RAD51, UvsX, RecA, онкотерапия, рекомбиназная полимеразная амплификация, метод оптического захвата, нуклеопротеиновый филамент

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Introduction

Homologous recombination is a key mechanism for maintaining genome stability, enabling cells to repair DNA double-strand breaks, restart stalled replication forks, and exchange genetic material during meiosis [1, 2]. Recombinases play a central role in homologous recombination; they are highly conserved DNA-binding proteins that coat single-stranded DNA (ssDNA), forming a nucleoprotein filament. This filament searches for a homologous sequence on another DNA molecule and promotes strand invasion [3, 4].

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The eukaryotic recombinase RAD51, the bacterial recombinase RecA, and the phage recombinase UvsX belong to the same superfamily, which is represented in all domains of life [5, 6]. The phage recombinase UvsX is functionally closer to the bacterial RecA protein, participating in homologous recombination, recombination-dependent replication, and DNA repair in infected host cells [7, 8]. All members of the superfamily contain a conserved RecA-like ATPase domain with Walker A and Walker B motifs, which are involved in ATP binding and hydrolysis, regulating the assembly, stability, and dissociation of the nucleoprotein filament [5, 9–12].

RecA is the most thoroughly studied member of the family. The structure of its helical filament, intermonomer contacts, and monomer structure are known at near-atomic resolution owing to XRD analysis and cryo-electron microscopy [3, 9]. The functions of RecA in the SOS response, homologous recombination, replication fork restart, horizontal gene transfer, and biofilm formation are well characterized [13–18]. The DNA-dependent ATPase activity of RecA was measured: its k_{cat} values are 30 min^{-1} for the RecA filament on ssDNA and 20 min^{-1} on double-stranded DNA (dsDNA) [19].

Human RAD51 is the focus of considerable attention because increased expression of this recombinase in tumors confers resistance to DNA-damaging chemotherapeutic agents, making this protein a promising candidate for targeted therapy [20–22]. The ATPase activity of human RAD51 was characterized in [23], where it was shown that the catalytic constant k_{cat} is 0.21 min^{-1} in the presence of ssDNA and 0.07–0.11 min^{-1} in the presence of dsDNA.

The UvsX recombinase of T-even phages is substantially less studied than bacterial or eukaryotic homologs, although interest in its properties has increased considerably in recent years due to its use in recombinase polymerase amplification (RPA) [24, 25]. During RPA, UvsX forms nucleoprotein filaments on single-stranded primers and mediates their invasion into a double-stranded DNA template, allowing DNA polymerase to begin synthesis of a new strand without thermal denaturation of the duplex. The highest activity of UvsX among homologs makes this enzyme preferable for use in RPA. For example, according to the literature, the catalytic constant k_{cat} of hydrolysis for UvsX reaches 240–500 min^{-1}, which is an order of magnitude higher than the corresponding value for the bacterial RecA protein [26, 27].

The nucleoprotein filament formed by recombinases is a key structural unit of homologous recombination, providing homology search and strand invasion into donor DNA [28]. Binding of recombinases to DNA leads to significant lengthening of the molecule (approximately 1.5-fold) compared with B-form DNA and to changes in its mechanical properties, including stiffness and elasticity [9, 29–31].

Optical tweezers allow these structural transformations to be monitored in real time at the level of single DNA molecules, since changes in their length are measured under controlled external tension.

This work presents data on the assembly kinetics of nucleoprotein filaments of human RAD51 and bacteriophage T6 UvsX on dsDNA at different applied tensions. The results were obtained using optical tweezers.

Materials and methods

DNA construct. Linear dsDNA, 11,100 base pairs (bps) long, was used for single-molecule DNA experiments. The molecules had an identical natural sequence and carried biotin modifications at both ends for specific attachment to streptavidin-functionalized microspheres. A detailed procedure for obtaining and purifying such DNA constructs is described in [12, 32].

Preparation of purified proteins. Recombinant human RAD51 and bacteriophage T6 UvsX proteins were obtained by expression in *Escherichia coli* strains BLR(DE3)pLysS for RAD51 and Rosetta(DE3) for UvsX. Plasmids encoding the corresponding proteins fused to a polyhistidine tag were used. Cells were grown in Lysogeny broth (LB) medium to an optical density of 0.6, induced with isopropyl-D-1-thiogalactopyranoside (IPTG), and additionally incubated at 37 °C for 3 h. The target protein was purified from the resulting cell biomass by affinity chromatography on Ni-NTA agarose (NTA is nitrilotriacetic acid). The cell lysate was loaded onto the column in a buffer with 50 mM imidazole, after which the protein was eluted with a stepwise imidazole gradient. The resulting preparations had a purity of at least 95%, as confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Purified proteins were stored at −80°C in a buffer supplemented with 10% glycerol. A detailed protein purification procedure is described in [33, 34].

Laser tweezers setup. The unique Laser Tweezers setup, assembled based on a non-inverted Axio Imager.Z1 microscope (Carl Zeiss), was used for the experiments. The setup allows for optical trapping with two independent traps simultaneously by splitting the laser radiation (Nd:YAG, $\lambda = 1064$ nm, Spectra Physics, model BL-106C) by a polarizing beam splitter into two components polarized in mutually perpendicular directions. The beams were focused using an immersion objective with a high numerical aperture (NA = 1.25). To move the trap across the sample plane, the beam was directed through a mirror mounted on a piezoelectric positioning stage.

The experimental setup was supplemented with a u-Flux multi-channel microfluidics system (LUMICKS B.V.) for laminar flow and buffer exchange around the sample. The microfluidic chamber was mounted on a motorized microscope stage with three-axis positioning. Images were recorded with an iXon Ultra 897 camera (Andor Technology) with an electron-multiplying CCD array. Video data processing to determine the distance between microspheres and the corresponding DNA stretching force was performed in real time by specialized software we developed in the LabVIEW environment. The stiffness of the optical traps was calibrated from the viscous drag force experienced by a trapped microsphere moving at a given speed in a liquid medium of known viscosity.

Single-molecule DNA experiments

To perform DNA manipulations, microspheres and the DNA molecule attached between them were transferred between channels with laminar flows. Streptavidin-functionalized polystyrene microspheres 2.1 μm in diameter were captured in two independent optical traps in the first channel of the microfluidic system. These microspheres were then transferred to the second channel containing a DNA solution at a concentration of 30 pM. Under these conditions, one DNA molecule was attached to the microspheres via biotin–streptavidin bonds at its ends, thus fixing the DNA between two microspheres. After such fixation, this system was pre-stretched to a force of 10–20 pN in a channel containing only buffer solution to check the integrity of the molecule and exclude multiple attachments. Next, the DNA fixed between the microspheres was transferred to the fourth channel of the microfluidic system containing RAD51 or UvsX recombinase at a concentration of 300 nM.

The buffer solution used had the following composition:

40 mM Tris-HCl (pH 7.5); 0.02% BSA; 1 mM DTT; 5 mM $MgCl_2$; 2 mM ATP.

The assembly of the nucleoprotein filament on the DNA molecule was recorded under these conditions. Measurements were performed in force-clamp mode in the range of 3–12 pN at 22 °C. Nucleoprotein filament assembly was monitored in real time by the increase in the length of the DNA molecule upon complex formation.

The optical trapping method used in this work allows manipulating single biomolecules and monitoring changes in their mechanical properties by controlling the tension force (in the range from fractions of a piconewton to hundreds of piconewtons) and recording the extension of the molecule with nanometer resolution.

Results and discussion

The binding kinetics of RAD51 and UvsX recombinases to DNA was studied using 11,100 bps long dsDNA. A single DNA molecule was trapped by combining the optical tweezers method with the microfluidic system (Fig. 1,*a*). At a fixed tension force, the DNA molecule has an almost constant length determined by entropic and enthalpic contributions to the elastic response. The experimentally obtained dependence of tension force on extension for the DNA construct used is shown in Fig. 1,*b*.

Transfer of the trapped DNA molecule into the channel containing RAD51 recombinase led to nucleoprotein filament assembly. A constant tension force was applied to the sample during incubation, and a change in length was recorded. Fig. 2 shows representative assembly curves of the RAD51 filament obtained at tension forces $F = 3$ pN, 6 pN and 12 pN. The assembly kinetics of the RAD51 filament were only weakly sensitive to variations in the applied tension. In all cases, the complex length reached saturation within 60–80 s.

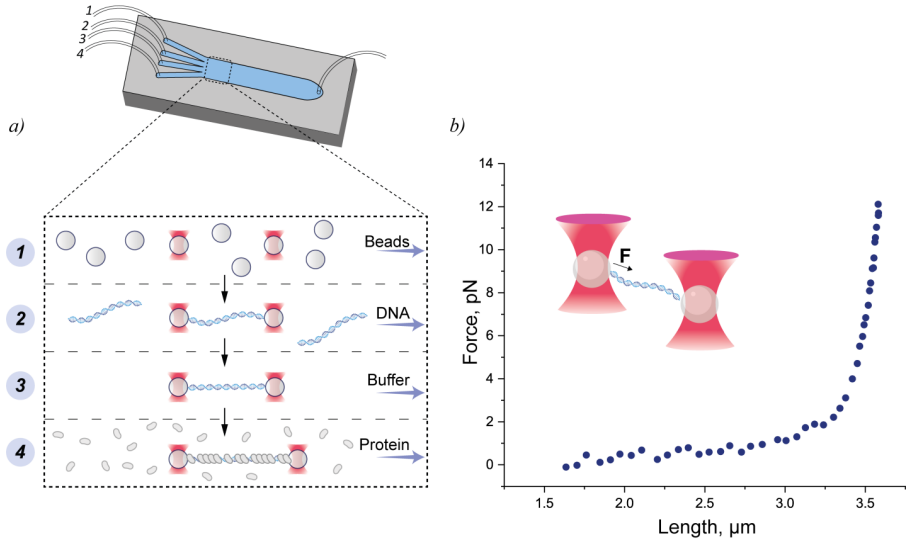


Fig. 1. Graphical representation of single-molecule DNA experiments: schematic image of microfluidic chamber with four laminar flows *1–4* (*a*); example of obtained dependence of tension *F* (pN) on the length of the DNA molecule used in this work (*b*)

The final values of the filament length were

4.82 ± 0.09 μm ($N = 4$) for $F = 3$ pN;

5.02 ± 0.04 μm ($N = 6$) for $F = 6$ pN;

4.99 ± 0.11 μm ($N = 4$) for $F = 12$ pN.

It is useful to compare the results obtained on the final DNA elongation upon assembly of the RAD51–DNA filament with data known from structural studies. According to structural data, DNA in the primary site of the nucleoprotein filament is elongated approximately 1.5-fold relative to the contour length of B-form DNA [5, 35]. The contour length of the DNA molecules used (11,000 bps) is about 3.7 μm. Due to entropic factors, the DNA length recorded as the end-to-end distance was shorter than the contour length and amounted to approximately 3.5 μm at $F = 6$ pN. A 1.5-fold increase of this value gives an estimate of 5.25 μm for the length of filaments formed on the double-stranded DNA used, which agrees with the final DNA elongation of 1.5 μm recorded in the experiments. This result indicates a fairly high degree of DNA coverage by RAD51 recombinase in the formed nucleoprotein filaments.

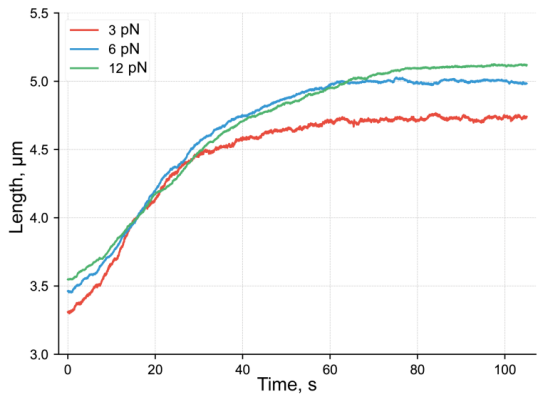


Fig. 2. Representative kinetic curves of RAD51 filament assembly on double-stranded DNA (dsDNA) at three different tension forces

Similarly, the assembly kinetics of UvsX filaments were studied. In contrast to RAD51, complete assembly of the nucleoprotein filament was observed only upon application of force $F = 12$ pN (Fig. 3,*a*). At $F = 3$ pN, no DNA elongation was observed during incubation in the UvsX channel, whereas at $F = 6$ pN only a limited increase in length was recorded, which did not reach a stationary value (during the observation time) corresponding to a 1.5-fold DNA elongation (Fig. 3,*b*).

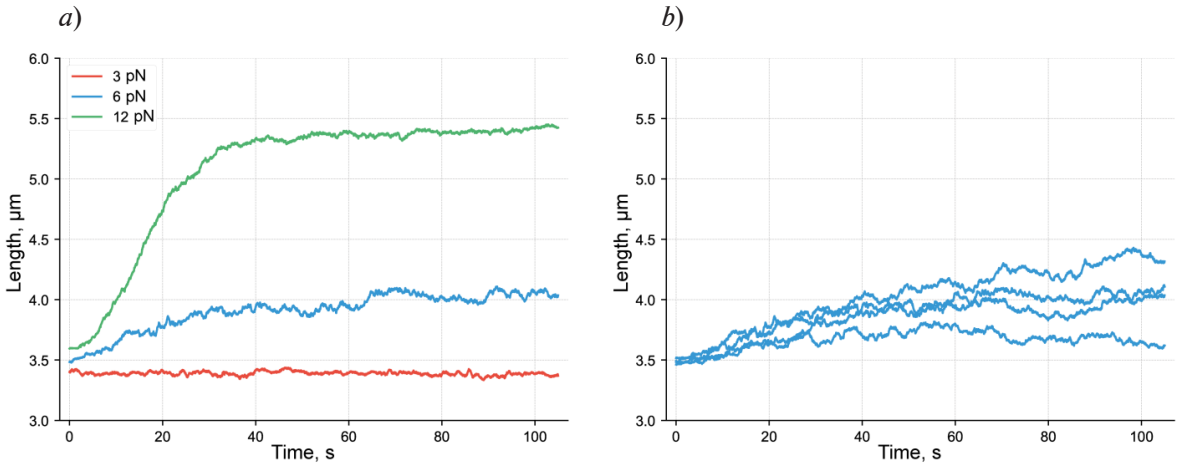


Fig. 3. Observations of UvsX filament assembly on dsDNA: representative assembly curves at three F values, pN: 3, 6, and 12 (*a*); UvsX filament assembly curves at $F = 6$ pN (*b*)

At $F = 12$ pN, the complex length reached saturation within 30–40 s, and the final length was 5.4 ± 0.1 μm ($N = 5$). This value is slightly higher than those observed at the same force for RAD51, but it also agrees with an approximately 1.5-fold DNA elongation. The exact filament length recorded in experiments using optical tweezers is determined, in addition to the tension force, by a number of factors, including the degree of DNA coverage by protein, the helix pitch of the filament, the number of monomers per helix turn, the persistence length, and the local presence of regions in an inactive conformation associated with ATP hydrolysis. Nevertheless, structural studies show that filaments formed by recombinases are characterized by DNA elongation of approximately 1.5-fold compared with the length of B-form DNA [9, 30, 31]. The observation of complexes with a greater length, for example at $F = 12$ pN, may indicate the assembly of UvsX filaments with a higher degree of DNA coverage than that of RAD51 filaments under these conditions.

Despite the prevailing opinion about the lower activity of RAD51 recombinase compared with RecA and UvsX, the results obtained indicate its ability to efficiently bind to dsDNA.

It is known from the literature that such binding is difficult for RecA recombinase from the Gram-negative bacterium *Escherichia coli*, typically observed only upon application of a sufficiently high external tension force exceeding 20 pN [32].

The activity of RAD51 recombinase in the cell is controlled by a number of mediator proteins, primarily at the stage of assembly of the nucleoprotein filament on ssDNA [5]. *In vitro*, RAD51 demonstrates increased efficiency of invasion of the 5′-terminal ssDNA fragment during catalysis of strand exchange, compared with the 3′-terminal fragment, which contrasts with the behavior of RecA, for which the 3′-terminal region is preferred [36]. It was shown that the efficiency of 3′-terminal fragment invasion is restored for RAD51 recombinase with duplex DNA with a single-stranded overhang.

In most models of homologous recombination, the assembly of the nucleoprotein filament on ssDNA in the presence of additional proteins, both competing for binding to ssDNA and stimulating the loading of the recombinase onto ssDNA, is considered the key initial stage. However, the natural substrate formed as a result of DNA processing and available for interaction with the recombinase is a hybrid DNA including a dsDNA region transitioning into a 3′-terminal single-stranded fragment. It can therefore be assumed that the assembly of a stable RAD51 filament on dsDNA can further increase (through cooperative interactions) the conformational stability of the RAD51 filament on ssDNA, promoting homologous recombination. The relationship between the conformational states of different regions of the filament formed on hybrid DNA was previously demonstrated for RecA recombinase [37].

UvsX recombinase is considered the most active and therefore the most popular choice for recombinase polymerase amplification systems. The catalytic constant of ATP hydrolysis for UvsX exceeds the corresponding value for RAD51 by three orders of magnitude. During infection of a cell by T-even bacteriophages, UvsX recombinase ensures recombination-dependent replication of multiple copies of the viral genome at late stages of infection, making it possible to synthesize hundreds of copies of phage DNA in a compressed time frame [38]. Uncontrolled binding of UvsX to dsDNA could interfere with replication processes under conditions of intensive phage DNA synthesis, as well as lead to unnecessary ATP consumption. In the case of RAD51, such interaction of the recombinase with dsDNA seems more likely and is not accompanied by significant ATP overconsumption due to the substantially lower ATPase activity of this recombinase. Moreover, RAD51 activity in eukaryotic cells is additionally controlled by a significantly wider set of regulatory proteins.

Conclusion

This work reports on the binding kinetics of RAD51 and UvsX recombinases at different external tensions in the range of 3–12 pN. The observed absence of UvsX binding to double-stranded DNA (dsDNA) without application of sufficient tension apparently reflects the evolutionary adaptation of the phage replication system. The different propensity of RAD51 and UvsX recombinases to bind dsDNA indicates possible alternative pathways for organizing recombination complexes *in vivo*.

The optical tweezers technique opened up the possibility for quantitatively characterizing the influence of mechanical stress on the formation and stability of recombinase complexes.

Single-molecule DNA studies allow direct testing of the influence of the type of DNA substrate and physical conditions on the assembly and stability of nucleoprotein filaments and provide a basis for a more detailed description of the molecular mechanisms of homologous recombination.

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