Electron microscopy

The reactions were performed in buffer R at 37 °C and had a final volume of 12.5 μl. To assemble the Rad51 presynaptic filament, M13mp18 (+) strand (7.2 μM nucleotides) and 1.3 μg Rad51 (2.4 μM) were incubated for 5 min. To test the effects of Srs2 and RPA, these proteins were added to the reaction mixtures containing the preassembled Rad51 presynaptic filament to final concentrations of 60 nM (Srs2) and 350 nM (RPA), followed by a 3-min incubation. In some cases, linear dsDNA (a 5.2-kilobase fragment derived from the pET24 vector) was also added with Srs2 and RPA to 7.2 μM base pairs, followed by a 5-min incubation. For electron microscopy, 3 μl of each reaction mixture was applied to copper grids coated with thin carbon film after glow-discharging the coated grids for 2 min. The grids were washed twice with buffer R and stained for 30 s with 0.75% uranyl formate. After air-drying, the grids were examined with a Philips Tecnai12 electron microscope under low-dose conditions. Images were recorded either with a charge-coupled device camera (Gatan) or on Kodak SO-163 films at × 30,000 magnification and then scanned on a SCAI scanner (Zeiss). The experiments shown in Fig. 4 were each independently repeated three or more times and at least 100 nucleoprotein complexes were examined in each experiment.

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Correspondence and requests for materials should be addressed to L.K. (krejci@uthscsa.edu) or P.S. (sung@uthscsa.edu).

The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments

Xavier Veaute*, Josette Jeusset†, Christine Soustelle*‡, Stephen C. Kowalczykowski§, Eric Le Cam† & Francis Fabre*

** CEA, DSV, Département de Radiobiologie et Radiopathologie, UMR217 CNRS/CEA, BP6, 92265 Fontenay aux Roses Cedex, France*
† Interactions Moléculaires et Cancer, UMR 81126 CNRS/IGR/UPS, Institut Gustave Roussy, Rue Camille Desmoulins, 94805 Villejuif Cedex, France
§ Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, University of California, Davis, California 95616-8665, USA

Homologous recombination is a ubiquitous process with key functions in meiotic and vegetative cells for the repair of DNA breaks. It is initiated by the formation of single-stranded DNA on which recombination proteins bind to form a nucleoprotein filament that is active in searching for homology, in the formation of joint molecules and in the exchange of DNA strands[1]. This process contributes to genome stability but it is also potentially dangerous to cells if intermediates are formed that cannot be processed normally and thus are toxic or generate genomic rearrangements. Cells must therefore have developed strategies to survey recombination and to prevent the occurrence of such deleterious events. In *Saccharomyces cerevisiae*, genetic data have shown that the Srs2 helicase negatively modulates recombination[2,3], and later experiments suggested that it reverses intermediate recombination structures[4–7]. Here we show that DNA strand exchange mediated *in vitro* by Rad51 is inhibited by Srs2, and that Srs2 disrupts Rad51 filaments formed on single-stranded DNA. These data provide an explanation for the anti-recombinogenic role of Srs2 *in vivo* and highlight a previously unknown mechanism for recombination control.

Several phenotypes (discussed below) conferred by the *srs2* deletion are suppressed by mutations that prevent formation of the Rad51 nucleofilaments[8,9]. Two hypotheses could explain this suppression: either Srs2 functions in replication and repair to prevent the formation of toxic recombination structures, or Srs2 disrupts dead-end recombination intermediates, possibly formed after the arrest of the replication fork, to allow repair through alternative pathways. This second proposition led us to ask whether purified Srs2 acts on preformed recombination structures.

Srs2 was expressed from a baculovirus vector in which *SRS2* was cloned in frame with a histidine tag at its amino terminus. We showed that the protein fusion expressed in yeast fully complements the sensitivity of *srs2*-deleted cells to radiation (data not shown).

‡ Present address: UMR2167 CNRS Centre de Génétique Moléculaire, Bâtiment 26, avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France.

After three steps of purification, summarized in Fig. 1a, the protein was nearly homogeneous (Fig. 1b).

Srs2 shares homologies with the bacterial UvrD, Rep[3] and PcrA helicases that extend beyond the helicase domains. These helicases belong to the SF-1 superfamily and are believed to translocate on single-stranded DNA (ssDNA), supported by their ssDNA-dependent ATPase activity[10]. We tested the ATPase activity of Srs2 with ssDNA or double-stranded DNA (dsDNA) as cofactors. Srs2 was previously shown to possess ATPase activity stimulated by M13mp18 ssDNA[11]. Because this ssDNA adopts secondary structures with dsDNA character[12], we used $(dT)_{55}$ ssDNA and a 55-mer blunt-ended dsDNA. The ATPase activity of Srs2 is highly stimulated by the ssDNA, whereas dsDNA has no effect (Fig. 1c). We then tested the helicase activity and found that it unwinds 3′ but not 5′ end-tailed duplex DNA, indicating a preferential 3′ → 5′ polarity, as previously shown[11]. We also found that it does not unwind a 24-mer blunt-ended dsDNA substrate (Fig. 1d). Taken together, these results show an exclusive requirement for ssDNA for the Srs2 activity.

To determine whether Srs2 inhibits recombination *in vitro*, we studied its effect on Rad51-promoted DNA strand exchange reactions involving ΦX174 linear dsDNA and circular ssDNA, using a protocol in which the ssDNA was incubated sequentially with Rad51 and the ssDNA binding factor RPA before addition of the dsDNA (Fig. 2a). In the first experiment, we examined whether Srs2 prevents DNA strand exchange. After formation of the Rad51–ssDNA nucleoprotein filament, linear dsDNA was added in the presence of increasing Srs2 concentrations. Samples were taken after 60 min of incubation. In the absence of Srs2, substantial levels of nicked circular duplex, the final DNA strand exchange product, and joint molecules were obtained (Fig. 2b, lane 1). When increasing amounts of Srs2 were introduced, a marked inhibition of strand transfer occurred and no nicked circular duplex was detected at Srs2 concentrations of 132 nM or higher (Fig. 2b, lanes 2–6, and Fig. 2c). This amount of Srs2 is about 1/100 that of Rad51, indicating that Srs2 acts catalytically. To further investigate the mechanism by which Srs2 inhibits DNA strand exchange, we studied its effect when being added during an ongoing DNA strand exchange reaction. A standard reaction, without Srs2, is shown in Fig. 2d,

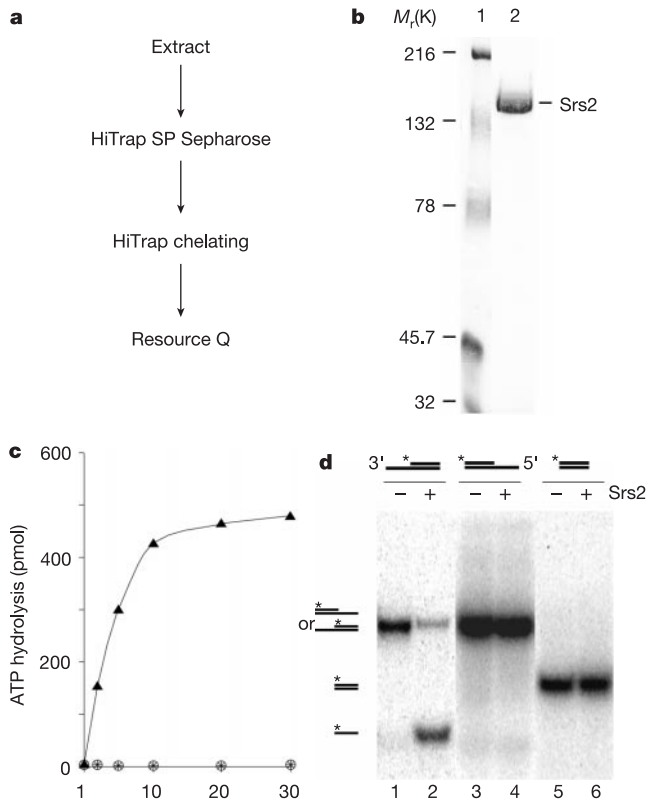

Figure 1 Purification, ATPase and helicase activities of Srs2. **a**, **b**, The Srs2 protein overexpressed in *Spodoptera frugiperda* cells was purified by three chromatographic steps (**a**) to near homogeneity as judged by analysis using 8% SDS–polyacrylamide gel electrophoresis and staining with Coomassie blue (**b**). **c**, Srs2 protein is a ssDNA-dependent but not a dsDNA-dependent ATPase. Purified Srs2 was incubated with [γ-^{32}P]ATP in the absence of DNA (asterisks) or in the presence of either (dT_{55}) (triangles) or a 55-mer blunt-ended dsDNA (circles). **d**, Srs2 protein unwinds a γ-^{32}P-labelled partial duplex DNA with a 3′ single-strand tail (lanes 1 and 2) but has no effect on either 5′ overhang (lanes 3 and 4) or blunt-ended duplex (lanes 5 and 6).

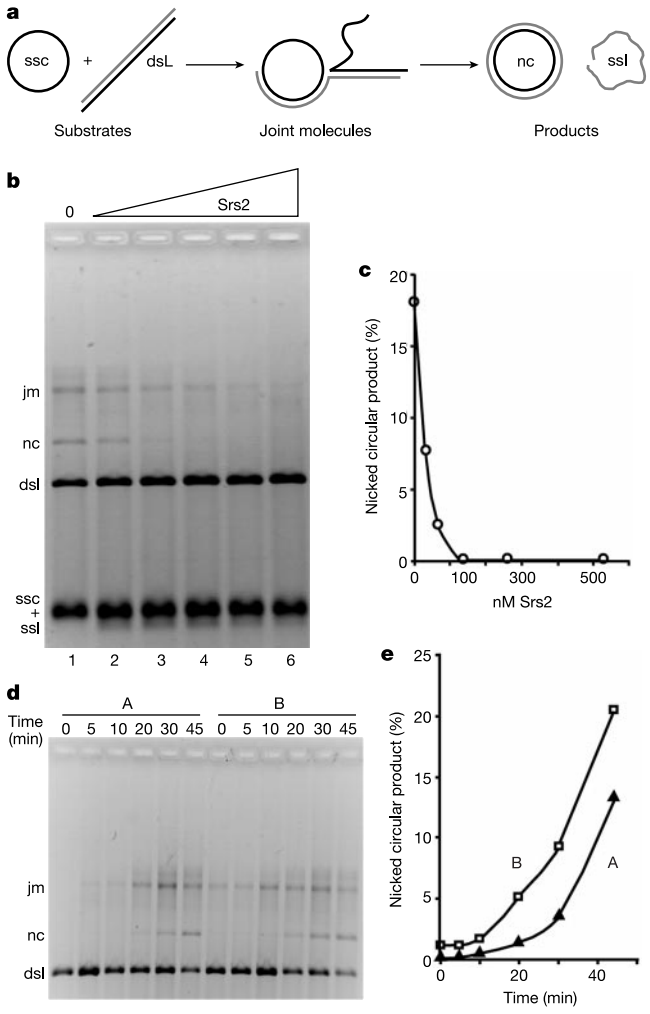

Figure 2 Srs2 inhibits DNA strand exchange catalysed by Rad51. **a**, Scheme of DNA strand exchange reaction. **b**, Increasing amounts of Srs2 were added simultaneously to linear dsDNA in a strand exchange reaction (lanes 1–6 correspond to 0, 33, 66, 132, 264 and 528 nM Srs2, respectively). **c**, Quantification of the reactions shown in **b**. **d**, Panel A: time course of a standard DNA strand exchange. Panel B: the reaction was started by addition of the linear dsDNA. Srs2 (264 nM) was added at various times after initiation (as indicated) and incubation was continued for 60 min. **e**, Quantification of the reactions shown in **d**. Abbreviations: dsl, double-stranded linear DNA; jm, joint molecules; nc, nicked circular double-stranded DNA; ssl, single-stranded linear DNA; ssc, single-stranded circular DNA.

panel A. Samples were recovered at different incubation times. In parallel, and at the corresponding times, Srs2 was introduced at a concentration of 264 nM and incubation was continued for a further 60 min (Fig. 2d, panel B). The comparison of results obtained in these two assays indicates no evidence for dissociation of the Rad51-made DNA joint molecules by Srs2. It might be that the helicase activity of Srs2 is not processive enough to unwind long dsDNA, which would have led to the observable dissociation of joint molecules. In fact, it seems that Srs2 freezes the reaction very quickly after its addition. The quantity of DNA strand exchange products for each time point is slightly higher in the samples to which Srs2 was added and incubated further (Fig. 2e). This is probably due to the time needed for Srs2 to act and block the reaction totally. One hypothesis accounting for this set of results is that Srs2 removes Rad51 from the ssDNA by means of its translocase activity. Elimination of the presynaptic filament would indeed stop the DNA strand exchange reaction.

Electron microscopy was used to reveal the possible disruptive effect of Srs2 on Rad51 nucleofilaments. Rad51 was first incubated with ΦX174 viral (+) strand for 20 min to assemble nucleoprotein filaments. All of the observed molecules were similar to that shown in Fig. 3a. When Srs2 (105 nM) was then added to the reaction and the incubation was continued for 2 min, Rad51 nucleoprotein filaments were disrupted to various extents. Of 532 molecules counted, 15% had partial nucleofilaments dismantled at different locations (Fig. 3b), whereas the others were revealed only by short nucleofilament structures. After 10 min of incubation with Srs2, no molecules were detected. The presence of naked DNA molecules was revealed by subsequent addition of the T4 gene 32 ssDNA-binding protein (Fig. 3c). The 452 molecules observed were all bound by T4 gene 32 protein, as shown in Fig. 3d, and were found at a concentration similar to that of the Rad51 nucleofilaments. T4 gene 32 protein alone does not displace Rad51 bound to ssDNA (580 molecules observed; data not shown). These experiments indicate that Srs2 displaces Rad51 from ssDNA. Last, we found that Srs2 does not disrupt Rad51 filaments formed on ΦX174 linear dsDNA (Fig. 3e, f). Thus, the removal of the Rad51 nucleofilaments by Srs2 occurs only on ssDNA, by a process that is probably linked to the translocation of Srs2 along ssDNA. This activity of Srs2 has also been recently shown by Sung *et al.*[13].

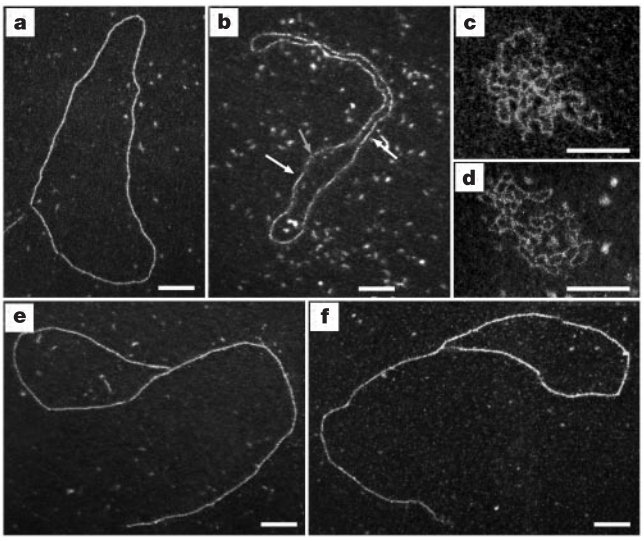

Figure 3 Disruption of Rad51 presynaptic filament by Srs2 as examined by electron microscopy. **a**, Rad51–ssDNA nucleoprotein filaments. **b**, Preformed Rad51–ssDNA filaments were incubated for 2 min with 105 nM Srs2. White arrows show intact Rad51 nucleoprotein filament and the grey arrow indicates regions of destabilized filament. **c**, ΦX174 viral (+) strand covered by T4 gene 32 protein. **d**, Preformed Rad51–ssDNA complexes were incubated for 10 min with 105 nM Srs2. T4 gene 32 protein was then added and incubation was continued for 10 min. **e**, Rad51–dsDNA nucleoprotein filaments. **f**, Rad51–dsDNA nucleoprotein filaments incubated for 10 min with Srs2 protein. Scale bars, 200 nm.

We next asked whether Srs2 is also active on RecA ssDNA nucleoprotein filaments. Srs2 was found to inhibit strand exchange and, by electron microcopy, to disrupt RecA–ssDNA nucleoprotein filaments (data not shown). Thus, Srs2 might have specificity for nucleoprotein filaments formed with the RecA/Rad51 class of recombinases. The fact that *SRS2*-deleted cells have strong phenotypes explainable by an abnormal persistence of Rad51 nucleofilaments most probably indicates that the disruption of nucleofilaments is an important role of Srs2 that is not replaced *in vivo* by other helicases. We now discuss some of these phenotypes.

The *RAD51*-dependent ultraviolet sensitivity of *srs2* cells[14] is explained by the binding of recombination proteins on ssDNA, formed before and/or after replication, generating toxic DNA structures if not processed by Srs2. Removal of the recombination proteins would be required to allow translesion synthesis. A role for Srs2 was also shown in cells expressing leaky alleles of *RAD51* and *RAD52*. These mutants are sensitive to ionizing radiation, a phenotype largely suppressed by the *SRS2* deletion[4–7]. In these cases the sensitivity of the mutants would be due to the disruption of the nucleofilament poisoned by the leaky proteins, thus preventing DSB repair. In the absence of Srs2, the leakiness of the system would permit repair. Similarly, we found that the cold sensitivity of *rad55*- or *rad57*-deleted cells to radiation[15] is suppressed by *srs2* (F.F. and X.V., unpublished data), indicating that, in those mutants, filaments are formed but disrupted by Srs2. It is important to note that Rad55 and Rad57 were absent from our assays *in vitro*, and it could be that the observed Srs2 activity *in vivo* relates to this absence.

The *srs2* mutant is also deficient in adaptation (escape from checkpoint arrest when cells suffer irreparable DSBs) and in recovery (the resumption of cell divisions after checkpoint arrest and completion of repair)[16]. It was proposed that Srs2 wipes out the checkpoint proteins from the DNA complex, eliminating the checkpoint signal. However, on the basis of the observation that the *srs2* defect in recovery is alleviated by *rad51* (ref. 16), the possibility that the role of Srs2 in recovery is to eliminate persistent Rad51 nucleofilaments that would otherwise trigger checkpoints is not totally excluded.

Last, Srs2 is essential in different mutant contexts, owing to the formation of toxic recombination intermediates. The synthetic lethality of *srs2* and *rad54* (ref. 17) is suppressed by *rad51* (refs 6, 18). The inactive Rad51 ssDNA nucleofilaments formed in *rad54* cells[19] would be toxic if not disrupted by Srs2. The *srs2 sgs1* growth defect also depends on the initiation of recombination[20], indicating that in *sgs1* cells potentially toxic recombination intermediates are formed, which are substrates for Srs2. The role of Sgs1 in recombination or/and replication is not yet clear[8]. Interestingly, Sgs1 is a helicase of the recQ family, which has orthologues in human cells that are responsible for the Bloom, Werner and a subset of Rothmund–Thomson syndromes, characterized by genomic instability and a predisposition to cancer[21]. These phenotypes might be related to unrestrained initiation of recombination that can lead to chromosomal instability. It was indeed shown that Wrn cellular phenotypes depend on Rad51-promoted recombination[22]. Our results should help us to understand the molecular basis of these syndromes.

Genetic data show that Srs2 is triggered by DNA lesions and by mutations affecting the structure and/or the activity of Rad51 nucleofilaments. Our finding supports the view that Srs2 disrupts nucleofilaments essentially when subsequent recombination steps are impaired. A close association between Srs2 and the recombination machinery is therefore predicted. □

Methods

Construction of recombinant baculovirus

Plasmid p14HB was constructed by deletion of the *Hin*dIII fragment of p14 (ref. 3) followed by the introduction of a new *Bam*HI site upstream of the *SRS2* open reading frame. The *Bam*HI–*Eco*RI fragment of p14HB containing the *SRS2* gene was cloned into pBacPAK-His1. Recombinant baculovirus was constructed as recommended by the manufacturer (Invitrogen). The recombinant baculovirus encodes the Srs2 protein with 17 additional amino acid residues (MGHHHHHHVVDKLGSQM) fused to its N-terminal methionine.

Purification of the recombinant Srs2 protein

Sf9 (*Spodoptera frugiperda*) cells were infected with the recombinant baculovirus at a multiplicity of infection of 10. After incubation for 50 h, cells were harvested, resuspended at 3×10^7 cells ml^{-1} in 50 mM phosphate buffer pH 7.2, 130 mM NaCl, 1 mM 2-mercaptoethanol, Triton X-100 × 1, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 2 mM benzaminide, 2 μM pepstatin, 5 μg ml^{-1} leupeptin and disrupted by sonication. The extract was cleared by centrifugation at 40,000 r.p.m. for 1 h. After dilution to achieve a final concentration of 100 mM NaCl and 10% glycerol, it was applied on a HiTrap SP Sepharose HP column (Amersham Biosciences) equilibrated with buffer A (50 mM phosphate buffer pH 7.2, 100 mM NaCl, 1 mM 2-mercaptoethanol and 10% glycerol). The column was eluted with a 60-ml linear gradient of 100–1,000 mM NaCl in buffer A. Fractions containing Srs2 were pooled and loaded on a HiTrap chelating column (Amersham Biosciences) equilibrated with 50 mM phosphate buffer pH 7.2, 500 mM NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol and 10% glycerol. The column was then washed with buffer B (20 mM phosphate buffer pH 7.8, 50 mM NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol and 10% glycerol). After elution with a 60-ml linear gradient of 20–400 mM imidazole in buffer B, fractions containing Srs2 were pooled and applied to a Resource Q column (Amersham Biosciences) equilibrated with buffer B without imidazole. The column was eluted with a 10-ml linear gradient of 50–600 mM NaCl. Highly purified Srs2 protein eluted at ~200 mM NaCl and was stored at −75 °C. Identity of the Srs2 protein was verified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (data not shown).

ATP hydrolysis assay

The Srs2 protein (6 nM) was incubated at 37 °C with DNA cofactor (4.53 μM nucleotides), 40 μM [γ-^{32}P]ATP in 25 μl reaction buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 7 mM $MgCl_2$, 5 mM dithiothreitol, 250 μg ml^{-1} BSA) and stopped by adding 20 mM EDTA pH 8. The level of ATP hydrolysis was measured as described[23].

Helicase assay

The substrates used to determine the directionality of the Srs2 activity consisted of partial duplex DNA annealed over 24 nucleotides with a single-strand tail of 21 nucleotides. Polarity 3′ → 5′ was checked with oligonucleotide P2 (5′-ACCTACCTCTGATTCGAAAC CTTC-3′) annealed with oligonucleotide P1 (5′-TGAAGGTTTCGAATCAGAGGTAGG TGCCCGGCCGGATAAATGCGCGTATTCCTGGT-3′). The opposite polarity was checked with oligonucleotide P3 (5′-ACCAGGAATACGCGCATTTATCCG-3′) annealed with P1. Blunt-ended duplexes were obtained by annealing P2 with the complementary oligonucleotide (P4). P2 and P3 were 5′ end-labelled with [γ-^{32}P]ATP and polynucleotide kinase. DNA substrate (20 fmol) was incubated at 30 °C with 121 nM Srs2 in 50 mM Tris-HCl pH 7.6, 7 mM $MgCl_2$, 2 mM ATP, 5 mM dithiothreitol and 25 μg ml^{-1} BSA for 30 min. The reaction products were resolved by electrophoresis on a 12% non-denaturing polyacrylamide gel in Tris-borate-EDTA buffer.

DNA strand exchange reaction

Reactions (10.5 μl) contained 42 mM MOPS pH 7.2, 3 mM magnesium acetate, 1 mM dithiothreitol, 20 mM NaCl, 25 μg ml^{-1} BSA, 2.5 mM ATP, 33 μM (nucleotides) ΦX174 viral (+) strand and 11 μM Rad51 protein. After incubation at 37 °C for 15 min, 1.1 μM RPA was added and incubation was continued for 5 min. Strand exchange was started by the addition of 1 μl (33 μM nucleotide final concentration) of *Pst*I-linearized ΦX174 dsDNA, 4 mM spermidine and variable amounts of Srs2 protein. After incubation, the samples were deproteinized and analysed by electrophoresis in 0.8% agarose gel in Tris-acetate-EDTA buffer. The gels were stained with ethidium bromide and quantified with ImageQuant software.

Electron microscopy

Rad51 filaments on ssDNA or dsDNA were formed by incubation of 6.48 μM Rad51 protein in DNA strand exchange buffer without BSA with 11 μM (nucleotides) ΦX174 viral (+) strand or *Pst*I-linearized ΦX174 replicative-form DNA for 20 min at 37 °C. Srs2 (105 nM final concentration) was then added for 10 min at 37 °C. To detect the presence of naked ΦX174 viral (+) strand after the action of Srs2, T4 gene 32 protein (0.65 μM) was subsequently added for 10 min. The reaction mixtures were diluted 1:50 in 10 mM Tris-HCl pH 7.5, 50 mM NaCl and 5 mM $MgCl_2$ without any chemical fixation, and analysed by electron microscopy as described previously[24].

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Correspondence and requests for materials should be addressed to X.V. (xavier.veaute@cea.fr) or F.F. (francis.fabre@cea.fr).