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A Rad51 Presynaptic Filament Is Sufficient to Capture Nucleosomal Homology during Recombinational Repair of a DNA Double-Strand Break

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Abstract

Repair of chromosomal DNA double-strand breaks by homologous recombination is essential for cell survival and genome stability. Within eukaryotic cells, this repair pathway requires a search for a homologous donor sequence and a subsequent strand invasion event on chromatin fibers. We employ a biotin-streptavidin minichromosome capture assay to show that yRad51 or hRad51 presynaptic filaments are sufficient to locate a homologous sequence and form initial joints, even on the surface of a nucleosome. Furthermore, we present evidence that the Rad54 chromatin-remodeling enzyme functions to convert these initial metastable products of the homology search to a stable joint molecule that is competent for subsequent steps of the repair process. Thus, contrary to popular belief, nucleosomes do not pose a potent barrier for successful recognition and capture of homology by an invading presynaptic filament.

INTRODUCTION

Cells suffer from DNA damage due to exogenous stresses that are present in their environment and endogenous stresses that are generated by metabolism (Allard et al., 2004). Chromosomal DNA double-strand breaks (DSBs) are one of the most severe types of DNA damage with respect to preservation of genomic integrity. If left unrepaired, DSBs can cause genomic instability in the form of chromosome loss or rearrangements, compromising faithful transmission of genetic information and eventually leading to apoptosis or carcinogenesis.

The *RAD52* group of genes mediates homologous recombinational repair (HRR) of DSBs in organisms ranging from yeast to human (Paques and Haber, 1999). The biochemical steps of HRR have been well-established in both prokaryotic and eukaryotic systems (Bianco et al., 1998; Sung et al., 2003). First, the 5′ ends of DNA that flank the break are processed by an exonuclease to create single-stranded DNA tails. Next, a recombinase (RecA in prokaryotes

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SUPPLEMENTAL DATA

The Supplemental Data include one table, Supplemental Experimental Procedures, and eight figures and can be found with this article online at http://www.molecule.org/cgi/content/full/30/6/803/DC1/.

and Rad51 in eukaryotes) polymerizes onto these DNA tails to form a nucleoprotein filament that can search for a homologous duplex DNA. In some cases, this homology search can occur on a genome-wide level (Haber et al., 1991; Inbar and Kupiec, 1999; Richardson et al., 1998). The initial detection of homology is thought to form a transient, metastable DNA joint in which the 3′ end of the presynaptic filament is not engaged in base-pairing interactions. This metastable joint is considered paranemic in nature, as it requires protein-DNA interactions (e.g., RecA-DNA or Rad51-DNA) (Bianco et al., 1998; Sung et al., 2003). Such paranemic joints are believed to be the precursor for a stable plectonemic joint (Riddles and Lehman, 1985) in which the presynaptic filament fully base pairs with its complementary strand, displacing the noncomplementary strand and forming what is known as a D loop. The stability of the plectonemic joint does not require protein-DNA interactions, and the 3′ end of the presynaptic filament is engaged in Watson-Crick base pairing. Subsequent steps of HRR entail DNA synthesis to replace the missing information, followed by resolution of DNA intermediates to yield two intact duplex DNA molecules.

Although studies in yeast and mammals have provided a wealth of information on the detection, response, and repair of DSBs, study of DSB repair in the context of chromatin is still rather nascent (Peterson and Cote, 2004). The first level of chromatin organization is the nucleosome, which is formed by wrapping ~147 bp of DNA around a histone octamer. Nucleosomes are organized as long, linear arrays that fold into condensed fibers and are stabilized by linker histones and intra- and internucleosomal interactions (Hansen, 2002). In the case of DSB repair, the wrapping of DNA on the surface of the histone octamer and the formation of chromatin fibers are likely to constitute potent barriers both for the homology search and strand invasion steps of HRR. Consistent with this view, numerous studies have demonstrated that ATP-dependent chromatin-remodeling enzymes are recruited to DSBs in vivo (Chai et al., 2005; van Attikum et al., 2007). In this study, we directly investigate whether the search for homology and the initial capture of the homologous sequence can take place on the surface of nucleosomes in the absence or presence of remodeling enzymes in vitro. We report that yeast and human Rad51 presynaptic filaments are sufficient to locate the homologous sequence and form initial joints even on the surface of a nucleosome.

RESULTS

The initial product of a successful homology search is believed to be a transient, protein-stabilized joint. Previous studies have used filter binding assays (Riddles and Lehman, 1985) or electron microscopy (Christiansen and Griffith, 1986) to visualize these metastable joints with DNA substrates. Filter binding assays have also been used to follow RecA-catalyzed formation of metastable joints on a nucleosomal donor (Ramdas et al., 1991). However, a drawback of such filter binding assays is that the synapsis reactions must be treated with high salt so that nucleosomes are displaced and joints can be quantified. In addition, these studies used presynaptic filaments assembled on very large ssDNA molecules, and thus it is not clear whether the filter binding assay can detect synapsis between smaller presynaptic filaments that might be targeted to a nucleosome. To circumvent these problems, we developed a biotin-streptavidin capture assay (Figure 1A). In this assay, a nucleosomal donor is assembled with recombinant histone octamers that are deposited onto a plasmid that harbors nucleosome-positioning sequences. The octamers are engineered to contain a site-

specific biotin group on the C-terminal domain of histone H2A (see the Supplemental Experimental Procedures available with this article online). Presynaptic filaments are then assembled with purified recombinases (RecA, yeast or human Rad51) on ^{32}P-labeled 50–90 base oligonucleotides that are homologous to target sequences in the nucleosomal donor. After incubation of the presynaptic filaments with the biotinylated nucleosomal donor, products of a successful homology search are captured on streptavidin-coated magnetic beads and quantified by scintillation counting (Figure 1A). Because the biotin-streptavidin capture assay measures total DNA joints (i.e., stable and metastable joints), one half of all reactions are also deproteinized so that stable plectonemic joints can be quantified by gel electrophoresis and autoradiography.

The donor plasmid contains a head-to-tail array of ten 5S rDNA nucleosome-positioning sequences that flank a dinucleosome length E4 promoter (Figure 1B) (Ikeda et al., 1999). EcoRI restriction sites flank each 5S repeat, and thus EcoRI digestion and native gel electrophoresis allow quantification of nucleosomal occupancy (Figure 1C) (Carruthers et al., 1999). When chromatin reconstitutions were performed at a ratio of 0.35 histone octamers per 200 bp of plasmid DNA (R = 0.35), very few of the 5S repeats were nucleosomal. At a ratio of 0.5 octamers per 200 bp of plasmid (R = 0.5), 50%–60% of the 5S repeats were nucleosomal (Figure 1C, compare lanes 1, 2, and 3). And finally at a ratio of R = 0.7, >90% of the 5S repeats were nucleosomal (Figure 1C, compare lanes 1 and 4). We confirmed the predicted locations of the two centrally located nucleosomes by two independent methods. First, PstI, XbaI, and StyI (Figure 1B) restriction enzyme digestion was used to probe nucleosomal occupancy. As predicted, all three restriction sites were accessible when donors were assembled at an R = 0.35 or R = 0.5, whereas the StyI site was occluded in the plasmid assembled at an R = 0.7 (Figure S1 and data not shown). Additionally, we digested nucleosomal donors with micrococcal nuclease (MNase), which cleaves nucleosomal DNA preferentially within the linker DNA between nucleosomes. MNase digests were electrophoresed on agarose gels, and Southern blots were probed with ^{32}P-labeled oligo B, which hybridizes to E4 sequences that are predicted to be encompassed by a positioned nucleosome (Figure 1B). When this analysis was performed on donors assembled at R = 0.7, oligo B hybridized exclusively to mononucleosomal DNA (Figure 1D, lanes 1–3) and to an extensive nucleosomal ladder (Figure 1D, lanes 3–6). Importantly, no free DNA fragments smaller than 100 bases were detected (Figure 1D, compare lanes 1–6 with 8–11). In contrast, MNase analysis of donors assembled at R = 0.35 or R = 0.5 allowed detection of smaller DNA fragments that are diagnostic of free DNA (Figure S2). These data confirm that the position B on the E4 promoter sequence (Figure 1B) is fully encompassed by a nucleosome in donors assembled at R = 0.7, but this region is only partially occupied in donors assembled at R = 0.35 and R = 0.5.

Based on previous studies, we assembled presynaptic filaments on four different oligonucleotides that should allow discrimination between stable versus metastable joints (Christiansen and Griffith, 1986; Konforti and Davis, 1987). For instance, a presynaptic filament assembled on an oligonucleotide that is fully homologous to the donor (Figure 1B, inset, oligo A1) should lead to formation of stable, plectonemic joints. However, presynaptic filaments assembled on oligonucleotides lacking homology to the donor at the 3′ end (Figure 1B, inset, oligos A2 and A3) are expected to form primarily metastable joints, whose

stability requires protein-DNA interactions. On the other hand, a presynaptic filament that lacks homology to the donor (Figure 1B, inset, oligo A4) should not form joints, and it serves as a negative control. In addition, to determine if joints are more likely to form between nucleosomes rather than on the nucleosomal surface, we also assembled presynaptic filaments on oligonucleotides that are homologous to different positions on the chromatin donor (Figure 1B, arrows labeled A, B, and C). Specifically, at position A, much of the presynaptic filament has homology to the linker region between nucleosomes, and only the 3′ end of the filament is predicted to lie within a nucleosome. On the contrary, at position B, most of the filament has homology to a sequence that is predicted to be buried within a nucleosome and only the 3′ end of the filament lies at the nucleosomal edge. At position C, the entire presynaptic filament has homology to sequences that are encompassed by each of the 5S-positioning elements and thus should be entirely nucleosomal.

To validate the biotin-streptavidin capture assay, presynaptic filaments were first assembled using RecA recombinase and the panel of ^{32}P-labeled oligonucleotides. When the capture assay was applied to reactions using the donor that had few nucleosomes (R = 0.5), 35% to nearly 60% of the presynaptic filaments were captured in a 30 min reaction (Figure 1E and Figure S3). Formation of joints required nucleotide cofactor, RecA, and homology between the presynaptic filament and nucleosomal donor (Figure 1E, compare reactions with oligo A4 to other oligos, and Figure S3). Moreover, joints were efficiently formed with all types of RecA presynaptic filaments, including those that lack 3′ homology and which produce mainly metastable, protein-dependent joints (Figure 1E and Figure S4). In addition, joint formation was ~5-fold more efficient in the presence of a nonhydrolyzable analog of ATP, ATP-γS, which also enhanced formation of stable plectonemic joints as expected (Figure S5 and data not shown), (Hsieh et al., 1992; Menetski et al., 1990). Strikingly, RecA catalyzed formation of joints even when the nucleosomal donor was fully loaded with nucleosomes (R = 0.7) and the presynaptic filament was homologous to sequences located on the nucleosomal surfaces (Figure 1E, oligos B and C). However, consistent with our previous study (Jaskelioff et al., 2003), we observed that nucleosome assembly blocked formation of stable, plectonemic joints by RecA filaments (Figure S6). Thus, as predicted by earlier filter binding studies (Ramdas et al., 1991), nucleosomes are not potent inhibitors of RecA-mediated homology search, but nucleosomes do block formation of stable, protein-independent plectonemic joints.

Eukaryotic Recombinases Are Sufficient to Mediate a Homology Search on Chromatin

In contrast to RecA, previous studies have demonstrated that yeast or human Rad51 is not sufficient to catalyze significant levels of stable plectonemic joints on either a naked DNA template or on chromatin (Jaskelioff et al., 2003; Raschle et al., 2004; Van Komen et al., 2002). To determine whether yeast Rad51 is able to catalyze formation of protein-stabilized joints on nucleosomal donors, we performed biotin-steptavidin capture assays with donors that contained three levels of nucleosomal density (R = 0.35, R = 0.5, R = 0.7). In addition, assays were performed with the entire panel of presynpatic filaments, including positions B and C, which are located on the nucleosomal surface (Figures 1C and 1D). Surprisingly, yeast Rad51 was sufficient to catalyze formation of protein-stabilized joints on each of the nucleosomal donors and with all presynaptic filaments (Figures 2D and 2E). Like the case

for RecA, higher levels of joints were detected in the capture assay when a nonhydrolyzable analog of ATP, AMP-PNP, was used as the nucleotide cofactor (compare Figure 2C to Figures 3A and 3B). Notably, joints were homology, recombinase, and nucleotide cofactor dependent (Figures 2C–2E), and formation of joints reached a maximum by 15 min (Figure 2C). Strikingly, the efficiency of joint formation was insensitive to the level of nucleosome density, and moreover, all types of yRad51 presynaptic filaments, including the ones lacking homology at the 3′ end, formed joints with similar efficiencies (Figures 2D and 2E, compare oligos A2 and A3 with A1). Likewise, presynaptic filaments assembled with human Rad51 were also sufficient to capture homology with nucleosomal donors (Figure 2F). Thus, eukaryotic recombinases are able to carry out a successful homology search even when synapsis occurs on the nucleosomal surface.

Chromatin-Remodeling Enzymes Do Not Stimulate the Homology Search

Rad54 belongs to the SWI2/SNF2 subfamily of DNA-dependent ATPases, and biochemical studies indicate that Rad54 can function as ATP-dependent chromatin-remodeling enzyme (Alexeev et al., 2003; Alexiadis and Kadonaga, 2002; Jaskelioff et al., 2003). As expected, addition of yRad54 facilitated yRad51-mediated formation of plectonemic joints on nucleosomal donors (lanes 4–9, Figure 2B). Likewise, and consistent with previous studies (Jaskelioff et al., 2003; Raschle et al., 2004; Van Komen et al., 2002), yRad54 was also essential for formation of plectonemic joints on the naked DNA donor (lanes 1–3, Figure 2B). Surprisingly, addition of yRad54 did not enhance formation of yRad51-dependent joints in the biotin-streptavidin capture assays (Figures 3A and 3B; note that the inclusion of ATP in these reactions leads to levels of joint formation that are much lower than in reactions using AMP-PNP). In fact, addition of yRad54 decreased the overall efficiency of joint formation by as much as 2-fold, and this inhibitory effect was most apparent when presynaptic filaments were assembled with oligonucleotide A1 (Figure 3B). Moreover, addition of the SWI/SNF chromatin-remodeling enzyme did not stimulate formation of protein-dependent or -independent joints on nucleosomal donors (data not shown). We considered the possibility that yRad54 might stimulate joint formation at lower levels of nucleosomal density; however, yRad54 was also unable to stimulate joint formation on donors that contain fewer nucleosomes (R = 0.35 and R = 0.5; Figure 3A and Figure S8).

We investigated whether the remodeling activity of yRad54 or SWI/SNF might stimulate the homology search when presynaptic filaments are targeted to donor sequences that are predicted to be fully encompassed by the nucleosome. However, like the case for presynaptic filaments directed at position A, neither SWI/SNF nor yRad54 facilitated formation of joints by yRad51-presynaptic filaments targeted at positions B or C, which are on the surface of nucleosomes of the donor (Figure 3B and data not shown).

These data suggest a linear sequence of events that lead to formation of a stable plectonemic joint (D loop) on a nucleosomal donor: (1) Rad51 assembles onto ssDNA to form the presynaptic filament; (2) the presynaptic filament searches a donor for a homologous DNA duplex and forms an initial protein-dependent joint; and (3) the yRad54 ATPase converts this initial joint into a stable, protein-independent joint. This latter step may require a chromatin-remodeling role for yRad54. One prediction of this model is that the rate of

formation of stable, plectonemic joints should be enhanced by prior formation of the initial metastable joint. To test this hypothesis, we carried out two types of order-of-addition experiments (Figure 3C). In scheme 1, yRad54 was added to the yRad51 presynaptic filament prior to addition of nucleosomal donor. This reaction scheme is identical to our typical plectonemic joint assay (Figure 2B). In scheme 2, the yRad51 filament was preincubated with the nucleosomal donor in the absence of yRad54 to allow formation of the initial metastable joint molecule. yRad54 was subsequently added, and the rate of plectonemic joint formation was assayed. Strikingly, preformation of the metastable joint significantly enhanced the rate of stable joint formation following addition of yRad54 (Figure 3C). For instance, within 10 s of Rad54 addition, 20% of the maximum number of stable joints were formed. In contrast, nearly 2 min was required to form a similar amount of joints in the absence of the preincubation step. These data support a linear pathway in which the yRad51 presynaptic filament forms a metastable joint on the nucleosome surface that is subsequently converted to a stable plectonemic joint via the ATPase activity of yRad54.

DISCUSSION

Our study reveals that presynaptic filaments assembled with either prokaryotic or eukaryotic recombinases are sufficient to locate homologous sequences and form joints on the surface of a nucleosome. In this purified system, an ATP-dependent chromatin-remodeling enzyme is not required for the homology search process, nor does a remodeling enzyme increase the efficiency of this initial step. In contrast, the Rad54p ATPase promotes a subsequent step (formation of a plectonemic joint), which facilitates later steps in HRR.

The mechanism by which presynaptic filaments carry out the homology search in the dense, nucleoprotein environment of the nucleus (or bacterial nucleoid) is largely unknown. Several fundamentally different models for the homology search process have emerged over the years. First, linear diffusion or sliding of RecA presynaptic filaments was proposed in which the presynaptic filament binds to a dsDNA nonspecifically and then linearly diffuses or slides along the dsDNA to locate homology (Gonda and Radding, 1983). A second model is based on studies with both RecA and hRad51 and proposes that the homology search involves random three-dimensional collisions (Adzuma, 1998; Folta-Stogniew et al., 2004; Gupta et al., 1999).

Irrespective of a sliding or collision model, how is homology initially detected? Fluorescence resonance energy transfer analyses with hRad51 indicate that rapid exchange of A:T base pairs between the invading nucleoprotein filament and the duplex donor is key to allow the search for homology to proceed (Gupta et al., 1999). Likewise, kinetic studies using stopped-flow fluorescence and RecA have shown simultaneous switching of A:T bases and the formation of joints in which strand exchange has not been completed (Folta-Stogniew et al., 2004). These data suggest a model for the initial capture of homology in which DNA in the presynaptic filament aligns with the homologous duplex and bases from the donor rotate out of their stacked conformation to allow the formation of transient pairing interactions. These initial interactions are promoted by the extended structure of ssDNA within the presynaptic filament (Klapstein et al., 2004) and stabilized by RecA or Rad51.

Our data suggest that wrapping DNA onto a histone octamer may not have an adverse effect on such transient base flipping and that the presynaptic filament can sample homology on the nucleosomal surface. Once extensive homology is detected and the initial joint is formed, it is not clear if the Rad51 filament disrupts nucleosome structure. It seems likely that extensive alignment of the presynaptic filament with nucleosomal DNA will lead to DNA structures that would be incompatible with the wrapping of DNA around the octamer. Thus, the Rad51 filament may represent a previously unknown type of chromatin-remodeling enzyme. However, more extensive disruption of histone-DNA contacts (e.g., by Rad54) may be required to convert these transient interactions into stable joints. This sequence of events is consistent with recent studies that demonstrate homology-dependent chromatin remodeling by human Rad54 (Zhang et al., 2007).

For both RecA and Rad51, ATP hydrolysis leads to the turnover of presynaptic filaments (Chi et al., 2006; Cox, 2003). Indeed, we found that inclusion of ATP in the reactions led to more unstable plectonemic joints (Figure S5) and greatly diminished levels of joints detected by the biotin-streptavidin capture assay (Figures 3A and 3B and data not shown). In contrast, inclusion of a nonhydrolyzable analog of ATP, either ATPgS or AMP-PNP, stabilized both protein-dependent and protein-independent joints. In addition to the cycling of Rad51 due to ATP hydrolysis, we also found that the Rad54 ATPase led to a further decrease in the levels of joints detected by the biotin-streptavidin capture assay (Figures 3A and 3B). These results are consistent with the ability of Rad54 to displace Rad51 from DNA in an ATP-dependent reaction (Solinger et al., 2002). The initial metastable joints that are detected in the capture assay may be particularly prone to the displacement activity of Rad54.

Our results are consistent with previous studies in yeast that employed chromatin immunoprecipitation (ChIP) assays to monitor completion of the homology search process (Sugawara et al., 2003; Wolner and Peterson, 2005). These studies showed that the Rad51 recombinase could be detected by ChIP at the HML donor ~40 min after DSB formation, reflecting completion of a successful homology search. Likewise, and consistent with our in vitro studies, recruitment of Rad51 to the donor locus was also observed in strains that lacked a functional Rad54 ATPase. In contrast, Laurent and colleagues have reported that Rad51 cannot be detected at the donor locus in the absence of the SWI/SNF-remodeling enzyme (Chai et al., 2005). Paradoxically, *swi/snf* mutant strains are not extremely sensitive to DSB-inducing agents, suggesting that SWI/SNF is not generally required for HRR. Likewise, our in vitro studies indicate that the chromatin-remodeling activity of SWI/SNF does not generally enhance homology search on chromatin donors. One interesting possibility is that the role of SWI/SNF in HRR is restricted to situations in which the donor locus is embedded in highly condensed, heterochromatic chromatin structures, such as the SIR-dependent chromatin at HM donor loci. These condensed structures may restrict the intrinsic ability of the Rad51 presynaptic filament to capture homology, creating requirements for additional remodeling enzymes.

EXPERIMENTAL PROCEDURES

Reagent Preparation

DNA—Oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA) were 5′ end labeled with ^{32}P using γ-^{32}P -ATP and T4 polynucleotide kinase (New England Biolabs, Inc.; Beverly, MA). For oligonucleotide sequences, see Table S1. Plasmid CP943 (p2085S-G5E4) was prepared by alkaline lysis method, but caution was taken to minimize the incubation time in the alkaline buffer to maximize the yield of the supercoiled form and minimize the yield of nicked-circular form.

Proteins—Recombinant yRad51 was purified as described (Zaitseva et al., 1999). GST-yRad54 was expressed in yeast and purified as described (Solinger et al., 2001). Recombinant H2B, H4, and H2AS113C, H3C110A *Xenopus* histones were purified and biotinylated octamer was reconstituted as described earlier (Luger et al., 1999). For details about the biotinylation process, see the Supplemental Experimental Procedures.

Nucleosome Assembly—The circular nucleosomal donor was assembled by gradient salt dialysis of supercoiled plasmid CP943 (Ikeda et al., 1999) and biotinylated recombinant octamers as described (Logie and Peterson, 1999). Nucleosomes were reconstituted at different ratios of histone octamer per 200 bp of donor DNA (R value). Nucleosomal occupancy of the 5S repeats within the donor was evaluated by EcoRI analysis (Logie and Peterson, 1999; Carruthers et al., 1999). Nucleosomal occupancy within the central E4 sequence was analyzed by restricted MNase digestion of the donors followed by Southern hybridization. For details, see the Supplemental Experimental Procedures.

Joint Capture Assays—To form the presynaptic filaments, 1 μM of RecA or Rad51 was incubated with radiolabeled oligonucleotides (3 μM nucleotides) in 35 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 30 mM KCl, 1 mM DTT at 30° C for 5 min in 10 μl. As a nucleotide cofactor, 1 mM AMP-PNP, 0.3 mM ATP-g-S, or 2 mM ATP with an ATP regenaration system consisting of 20 mM creatine phosphate and 30 μg/ml creatine phospho-kinase was used as indicated. Then, 2 μl of naked DNA or nucleosomal donor at a final concentration of 33 nM DNA (plasmid molecules) was added and allowed to form joint molecules. Thus, the presynaptic filament and duplex donor DNA are present at equimolar ratios. Reactions were supplemented with an additional 12.5 mM $MgCl_2$ at the time of addition of the donor and then incubated at 30° C (for yRad51) or at 37° C (for RecA and hRad51) for the indicated time. In the reactions containing yRad54, 200 nM yRad54 (~1 yRad54 per presynaptic filament) was added during formation of presynaptic filaments after incubating the oligonucleotides with yRad51 for 2 min and then incubated for an additional 3 min. One half of the reaction mixture was deproteinized with 2% SDS and 2 mg/ml ProteinaseK for 5′ at 37° C, electrophoresed on a 0.9% agarose gel, and plectonemic joints detected by autoradiography (Jaskelioff et al., 2003). Percent plectonemic joints were calculated as the proportion of the homologous donor converted into radioactive joints.

To capture all joints (metastable and stable), half of the reaction mixture was added to 20 μl of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway) that had been preblocked in reaction buffer containing 1 μg/ml BSA and 1

mM sonicated salmon sperm DNA. After 5 min, joints were captured, washed three times with reaction buffer and unbound, and bound fractions counted in a scintillation counter. Percent total joints were calculated as proportion of bead-bound radioactivity to total (unbound + bound) radioactivity added into each reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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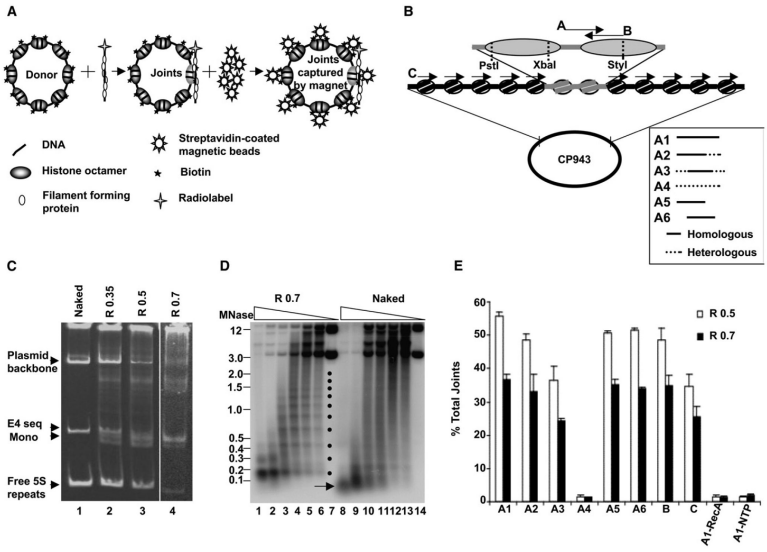

Figure 1. The Biotin-Streptavidin Capture Assay

(A) Schematic of the strategy for capturing initial joint molecules.

(B) Schematic of the nucleosomal, minichromosome donor. Blank ovals, 5S-rDNA nucleosome-positioning elements; gray ovals, putative positions of nucleosomes over central 400 bp E4 sequence; solid gray line, E4 sequence; broken black lines, sites for PstI, XbaI, and StyI; black arrows, oligonucleotides at positions A, B, and C. (Inset panel) Oligonucleotides at position A with varying amounts of homology to the donor. A1, 80 bases of homology; A2, 50 bases of homology followed by 30 bases of heterology at the 3′ end; A3, 50 bases of central homology flanked by 25 bases of heterology at 5′ end and 29 bases of heterology at 3′end; A4, 84 bases of heterology; A5, 50 bases of the homologous sequence from A2; A6, 50 bases of homologous sequence from A3. All oligonucleotides are depicted 5′ to 3′; B and C oligonucleotides have 87 and 80 bases of homology, respectively.

(C) EcoRI analysis of nucleosomal donors. Reconstituted minichromosome (R = 0.35, R = 0.5, or R = 0.7, lanes 2–4) or naked plasmid CP943 (lane 1) was digested with EcoRI, and products were separated on a 4% native polyacrylamide gel. Ethidium-stained gels are shown; lane 4 is from a separate gel. Mono, migration of 5S mononucleosome.

(D) Analysis of nucleosomal occupancy at position B of the donor. Reconstituted nucleosome (R = 0.7, lanes 1–7) or naked plasmid CP943 (lanes 8–14) was digested with increasing amounts of MNase, and purified products were separated by agarose gel electrophoresis followed by Southern hybridization using radiolabeled oligonucleotide B. Lanes 7 and 14 contain uncut purified DNA. DNA size markers in kilobases are shown at the left of the panel. Black arrowhead indicates free DNA fragments in lanes 8–11. Black dots indicate the banding pattern of MNase-sensitive sites due to nucleosome occupancy in lanes 1–6.

(E) Results of biotin-streptavidin capture assays using different RecA presynaptic filaments (see Figure 1B) with donors that are either subsaturated (R = 0.5) or saturated (R = 0.7) with nucleosomes. Reactions were incubated for 30 min and contained ATP-γ-S as nucleotide cofactor. Percent total joints were calculated as the proportion of bead-bound radioactivity to total (bound + unbound) radioactivity. Results are from at least three independent experiments; error bars indicate standard deviations.

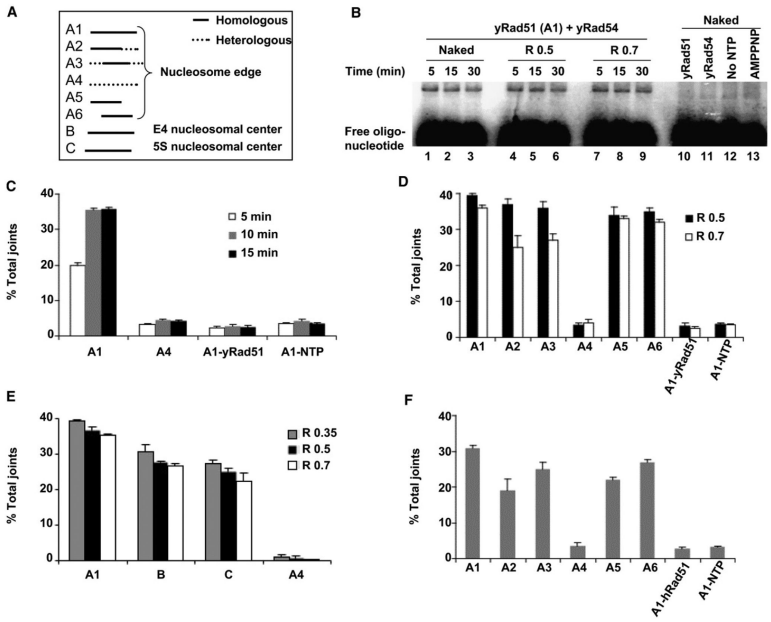

Figure 2. Yeast and Human Rad51 Are Sufficient to Capture Homology on Nucleosomes

(A) Schematic of oligonucleotides indicates different positions and different amounts of homology to the donor.

(B) Representative autoradiograph shows yRad51-mediated formation of plectonemic joints on naked (lanes 1–3) or nucleosomal donors (R = 0.5 or R = 0.7, lanes 4–9) as detected by agarose gel electrophoresis of deproteinized reaction products. yRad54 was added where indicated. Lanes 10–13 show formation of stable joints after 30 min. Lanes 12 and 13 contained both yRad51 and yRad54. All reactions contained oligo A1 and ATP, except where indicated.

(C) Biotin-streptavidin capture assays. Time course of yRad51-mediated formation of joints on the nucleosomal donor (R = 0.7).

(D) Biotin-streptavidin capture assays. Bar graph represents yRad51-mediated joint formation on nucleosomal donor with high (R = 0.7) and low (R = 0.5) nucleosomal occupancy. Oligonucleotides used for formation of presynaptic filaments are depicted on the x axis.

(E) Biotin-streptavidin capture assays. Bar graph represents yRad51-mediated joint formation at different positions on the nucleosomal donors with varying levels of nucleosomal saturation.

(F) Bar graph shows results from biotin-streptavidin assays with hRad51 presynaptic filaments and the R = 0.7 nucleosomal donor. Reactions in (C)–(F) were incubated for 20 min and contained AMP-PNP. Results in (C)–(F) are from at least three independent experiments; error bars indicate standard deviations.

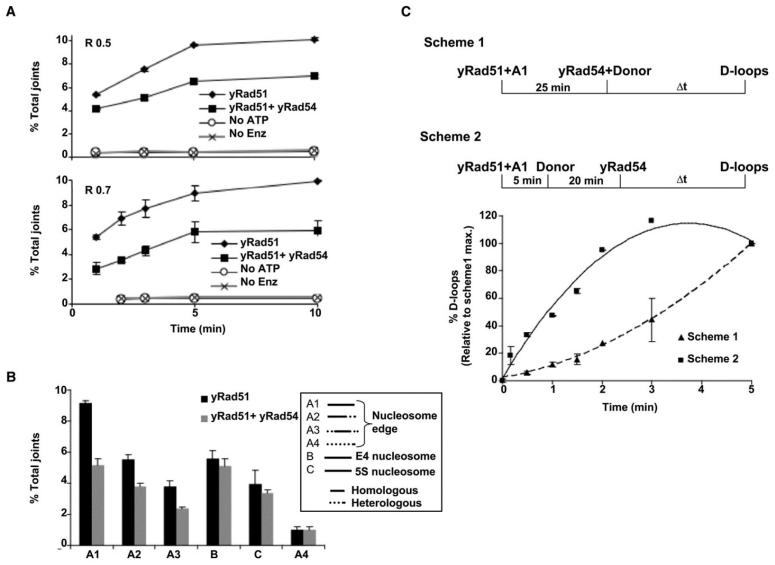

Figure 3. Rad54 Does Not Enhance Formation of Initial Joint Molecules

(A) Time course of yRad51-mediated formation of initial joints on R = 0.5 (top) or R = 0.7 (bottom) nucleosomal donors in the presence or absence of yRad54, as detected by the biotin-streptavidin capture assays using oligo A1. Reactions contained ATP, except where indicated, and hence lowered the efficiency of joint formation.

(B) Bar graph represents biotin-streptavidin capture assays with different yRad51 presynaptic filaments on the R = 0.7 donor. Grey bars denote reactions that also contained yRad54. Inset panel shows the different positions and varying amounts of homology of the presynaptic filaments.

(C) Top, schematic of timecourses. Bottom, quantification of plectonemic joints (D loops) assayed by agarose gel electrophoresis of the deproteinized reaction products and autoradiography. Amount of plectonemic joints detected after 5 min (scheme 1) was set to 100%. Reactions in (A)–(C) are carried out in presence of ATP. Results are from at least three independent experiments; error bars indicate standard deviations.