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Rad54 Protein Stimulates Heteroduplex DNA Formation in the Synaptic Phase of DNA Strand Exchange *via* Specific Interactions with the Presynaptic Rad51 Nucleoprotein Filament

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RAD54 is an important member of the *RAD52* group of genes that carry out recombinational repair of DNA damage in the yeast *Saccharomyces cerevisiae*. Rad54 protein is a member of the Snf2/Swi2 protein family of DNA-dependent/stimulated ATPases, and its ATPase activity is crucial for Rad54 protein function. Rad54 protein and Rad54-K341R, a mutant protein defective in the Walker A box ATP-binding fold, were fused to glutathione-*S*-transferase (GST) and purified to near homogeneity. *In vivo*, GST-Rad54 protein carried out the functions required for methyl methanesulfonate sulfate (MMS), UV, and DSB repair. *In vitro*, GST-Rad54 protein exhibited dsDNA-specific ATPase activity. Rad54 protein stimulated Rad51/Rpa-mediated DNA strand exchange by specifically increasing the kinetics of joint molecule formation. This stimulation was accompanied by a concurrent increase in the formation of heteroduplex DNA. Our results suggest that Rad54 protein interacts specifically with established Rad51 nucleoprotein filaments before homology search on the duplex DNA and heteroduplex DNA formation. Rad54 protein did not stimulate DNA strand exchange by increasing presynaptic complex formation. We conclude that Rad54 protein acts during the synaptic phase of DNA strand exchange and after the formation of presynaptic Rad51 protein-ssDNA filaments.

Keywords: DNA strand exchange; DSB repair; genetic recombination; heteroduplex DNA; Rad54

Introduction

DNA repair is an essential part of the cellular response to DNA damage. DNA double-strand breaks (DSBs) are lesions that affect both strands of a DNA double helix and are therefore particularly damaging. DSBs are the major genotoxic lesions induced by ionizing radiation (IR), which is commonly used in medical diagnosis and therapy. Furthermore, DNA replication, which occurs in all proliferating cells, was proposed to lead to DSBs (Seigneur *et al*., 1998; Sonoda *et al*., 1998). A better understanding of the mechanisms required for DSB repair is therefore important. Genetic analysis in *Saccharomyces cerevisiae* led to the identification of the *RAD52* group of genes that are involved in recombinational repair (Paques & Haber, 1999). In *S. cerevisiae*, DSBs are primarily repaired by homologous recombination using the DNA sequence of the sister chromatid or homolog to restore the DNA to its original condition in an intrinsically error-free way. Other pathways of DSB repair include single-strand annealing and end joining mechanisms that are error-prone (Paques & Haber, 1999). Mutations in *RAD52* group genes in *S. cerevisiae* result in defects in DNA damage repair, including extreme sensitivity to IR and alkylating agents like MMS as well as moderate sensitivity to UV radiation (Friedberg *et al*., 1995). In addition, defects occur in chromosome metabolism during

Abbreviations used: DSB, double-strand breaks; GST, glutathione-*S*-transferase; ss, single-stranded; ds, double-stranded; IR, ionizing radiation; UV, ultraviolet; MMS, methyl methanesulfonate; hDNA, heteroduplex DNA; 5-FOA, fluoroorotic acid; BSA, bovine serum albumin.

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vegetative growth and during meiosis (Paques & Haber, 1999). The high degree of conservation of the *RAD52* group genes among all eukaryotes indicates that the entire pathway is conserved throughout evolution (Paques & Haber, 1999).

Mutations in *RAD51, RAD52* and *RAD54* cause the most extreme DNA repair phenotypes in the *RAD52* group (Paques & Haber, 1999). The Rad51 protein shows homology to *Escherichia coli* RecA, the prototypic bacterial recombination protein (Bianco *et al*., 1998). *In vitro*, Rad51 protein forms nucleoprotein filaments on ssDNA in a fashion similar to RecA protein (Ogawa *et al*., 1993) in the presynaptic phase of homologous recombination, and mediates homologous pairing and DNA strand exchange *in vitro* (Sung, 1994; Sung & Robberson, 1995) in the synaptic phase. Further steps in recombination include branch migration and Holliday junction resolution (postsynaptic phase), which are poorly understood in eukaryotes. Rpa, the eukaryotic ssDNA binding protein, stimulates DNA strand exchange by removing secondary structures from ssDNA which leads to efficient presynaptic complex formation (Sugiyama *et al*., 1997). Synergistic actions of Rad51 and Rad52 proteins during DNA strand exchange have been demonstrated. Rad52 protein acts as a mediator between Rad51 protein and Rpa, affecting also the presynaptic phase of the reaction by overcoming inhibitory effects of Rpa in presynaptic filament formation (New *et al*., 1998; Shinohara & Ogawa, 1998; Sung, 1997a). Likewise, Rad55-Rad57 heterodimer overcomes the inhibitory effects of Rpa during the nucleation phase of Rad51 protein on ssDNA (Sung, 1997b).

Biochemical studies indicate a direct involvement of Rad54 protein during homologous pairing. The formation of D-loops (ssDNA pairing with a supercoiled dsDNA) by Rad51 protein is strongly stimulated by Rad54 protein (Mazin *et al*., 2000a,b; Petukhova *et al*., 1998; van Komen *et al*., 2000) or Rdh54/Tid1, a protein with significant sequence homology to Rad54 (Petukhova *et al*., 2000). Moreover, the addition of Rad54 protein to a Rad51/Rpa-mediated DNA strand exchange (circular ssDNA pairing with linear dsDNA) dramatically stimulated pairing and led to the formation of high molecular mass networks (Petukhova *et al*., 1998, 1999). These findings indicated that efficient homologous DNA pairing requires the cooperation between Rad51 and Rad54 proteins. Rad54 protein has been shown to change the topological conformation of dsDNA, which has been interpreted to be mediated by unwinding rather than by protein-constrained changes in DNA writhe or the introduction of plectonemic supercoils (Petukhova *et al*., 1998, 1999; Tan *et al*., 1999; Mazin *et al*., 2000a; van Komen *et al*., 2000). Rad54 protein does not exhibit DNA helicase activity in various assays (Petukhova *et al*., 1998; Swagemakers *et al*., 1998). It has been speculated that topological change introduced by Rad54 protein also stems from an ability to track along the DNA (van Komen *et al*., 2000). The detailed mode of action of Rad54 protein during homologous pairing is not known.

The Rad54 protein sequence exhibits significant sequence homology to the Snf2/Swi2 group of DNA-dependent/stimulated ATPases (Eisen *et al*., 1995). Its members have been implicated in transcriptional regulation (*SNF2/SWI2, MOT1*), recombinational repair (*RAD54*), and in other DNA repair pathways, e.g. in nucleotide excision repair (*RAD16*), in postreplicational repair (*RAD5*) and in strand-specific repair (*RAD26, ERCC6*) (Eisen *et al*., 1995). Like Rad54 protein, Snf2/Swi2, Mot1 and Rad26 are DNA-dependent/stimulated ATPases, but they do not exhibit DNA helicase activity (Auble *et al*., 1994; Guzder *et al*., 1996; Peterson & Tamkun, 1995; Petukhova *et al*., 1998; Swagemakers *et al*., 1998). Mutations in the ATP binding site (Walker A motif) of Rad54 protein lead to a non-functional protein (Clever *et al*., 1999; Petukhova *et al*., 1999). It was proposed that the conserved domain in the Snf2/Swi2 protein family members modulates protein-DNA interactions at the expense of ATP hydrolysis (Pazin & Kadonaga, 1997). It is also possible that the dissociation of protein-DNA complexes is achieved indirectly by local transient unwinding of the DNA (Mazin *et al*., 2000a; Petukhova *et al*., 1999; Tan *et al*., 1999; van Komen *et al*., 2000).

Here, we present results from biochemical studies which address the function of Rad54 protein in DNA strand exchange, focusing on specific interactions between Rad54 and the Rad51 nucleoprotein filament. Rad54 protein stimulated DNA strand exchange carried out by Rad51 protein and Rpa by specifically promoting the formation of joint molecules. This was accompanied by a concurrent increase in the amount of heteroduplex DNA (hDNA), resulting in joint molecules with an average hDNA length similar to joints formed in the absence of Rad54 protein. Rad54 protein had no effect in the formation of the Rad51:ssDNA presynaptic filaments but instead acted in conjunction with established filaments to stimulate homologous pairing and hDNA formation. DNA strand exchange by the *Escherichia coli* RecA protein was not stimulated by Rad54 protein, emphasizing the importance of specific interaction between Rad54 and Rad51 proteins during recombination.

Results

GST-Rad54 protein is biologically functional

To ascertain that the addition of the GST tag used to purify Rad54 protein did not interfere with Rad54 protein function, different functional assays were carried out. *rad54*Δ cells expressing plasmid-encoded GST-Rad54 protein survived MMS treatment as well as wild-type cells when plated on glucose-containing plates where the promoter is expressed at a low level (Figure 1(a), upper part).

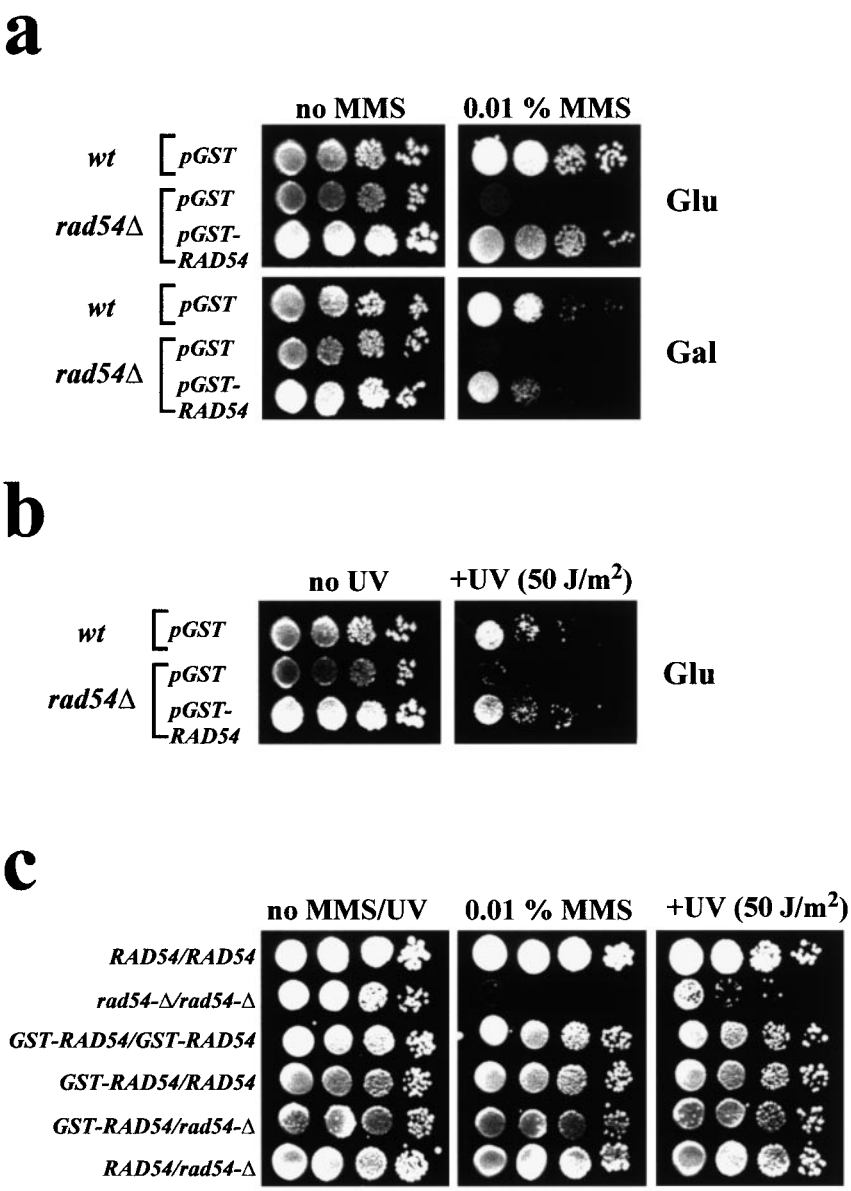

Figure 1. GST-Rad54 protein is biologically functional. (a) Complementation of MMS sensitivity. Serial dilutions of strains FF18733 (*wt*) and FF18973 (*rad54*Δ) containing plasmid pJN58 (*pGST*) or pWDH424 (*pGST-RAD54*) were spotted on full medium containing glucose (top) or galactose (bottom). (b) Complementation of UV sensitivity. The same strains as in (a) were analyzed as above on plates containing glucose. (c) Transplaced *GST-RAD54* is biologically functional. *GST-RAD54* fused to the native *RAD54* promoter was transplaced into the original chromosomal position and strains were assayed for DNA repair-related phenotypes. Serial dilutions of strains WDHY669 (*wt*), WDHY947 × WDHY949 (*rad54*Δ), WDHY1272 × WDHY1273 (*GST-RAD54/GST-RAD54*), WDHY1273 × FF18733 (*GST-RAD54/RAD54*), WDHY1273 × WDHY949 (*GST-RAD54/rad54*Δ) were analyzed.

When GST-Rad54 protein was overexpressed on plates containing galactose, the complementation was not complete (Figure 1(a), lower part). Deleterious effects of wild-type Rad54 protein overexpression have been observed before (Clever *et al.*, 1999), and the GST-Rad54 protein accumulates to levels five to ten times higher than the wild-type Rad54 protein used in that study (data not shown). Plasmid-borne GST-Rad54 protein also complemented the UV survival defects of *rad54*Δ cells (Figure 1(b)). To test if the fusion protein was able to restore the DSB repair defect of *rad54*Δ cells, we analyzed survival after induction of the HO endonuclease that delivers a single DSB in the genome of *S.cerevisiae*. The viability of the *RAD54* deletion strain expressing *GST-RAD54* restored viability to very near the level seen in the wild-type strain with the same plasmid (1.5 % *versus* 3 % in wild-type), whereas absence of *RAD54* resulted in very low survival in the presence of HO-endonuclease (<0.001 %).

To ensure that the GST-Rad54 fusion protein was able to perform its biological function also at the native protein level, the gene encoding GST-Rad54 protein was transplaced into the genome, so that the fusion gene was under the control of the endogenous *RAD54* promoter. Strains homozygous or heterozygous for *GST-RAD54* were as resistant to MMS and UV-radiation as wild-type strains and did not exhibit any obvious growth phenotype (Figure 1(c)). The *in vivo* protein levels of native Rad54 and GST-Rad54 were essentially identical, when analyzed by Western blotting using anti-Rad54 antibodies (data not shown). Moreover, no cleavage of the GST-Rad54 fusion protein to yield Rad54 protein lacking the GST tag could be detected on Western blots (data not shown). We conclude from these experiments that the GST-tag does not noticeably interfere with the *in vivo* DNA repair functions of Rad54 protein. From here on, we will refer to GST-Rad54 protein as Rad54 protein.

The dsDNA-dependent ATPase activity of Rad54 protein is quickly inactivated at 37°C, but is relatively stable at 30°C

Rad54 protein fused to GST was purified to near homogeneity (Figure 2). Rad54 protein showed a robust dsDNA-dependent ATPase activity with a turnover number of 1.95×10^3 min^{-1} at 30 °C (Figure 3(a)). This activity is comparable to the turnover number for a His(6)-Rad54 fusion protein reported previously (1.27×10^3 min^{-1} measured at 37 °C; Petukhova *et al.*, 1998) and for the human Rad54 protein that was purified with a polyhistidine and a hemagglutinin tag (0.8×10^3 min^{-1} measured at 30 °C; Swagemakers *et al.*, 1998). This indicates that the addition of GST does not greatly influence the ability of Rad54 protein to function as an ATPase, in accordance with the *in vivo* complementation studies.

The ATPase activity of Rad54 protein was completely dependent on the presence of dsDNA (herring sperm, poly(dA)·poly(dT)) and was abolished in the absence of DNA or in the presence of poly (dA) or poly(dT) ssDNA (Figure 3(a) and (b)). A mutant form of Rad54 protein with a mutation in the ATP binding fold (K341 → R) was also purified by the same method as the wild-type protein. Rad54-K341R protein displayed a greatly reduced ATPase activity (5.9 %) compared to the wild-type protein (Figure 3(a), inset), although this activity is significantly higher than the control without dsDNA (1.5 %; Figure 3(a), inset). This is consistent with previous observations with the same mutation in a different Rad54 fusion protein (Petukhova *et al.*, 1999). Rad54-K341R protein was used throughout this study to assess the importance of the ATPase activity of Rad54 protein. Taken together, these results also suggest that no significant contaminating ATPase activity was present in our preparation.

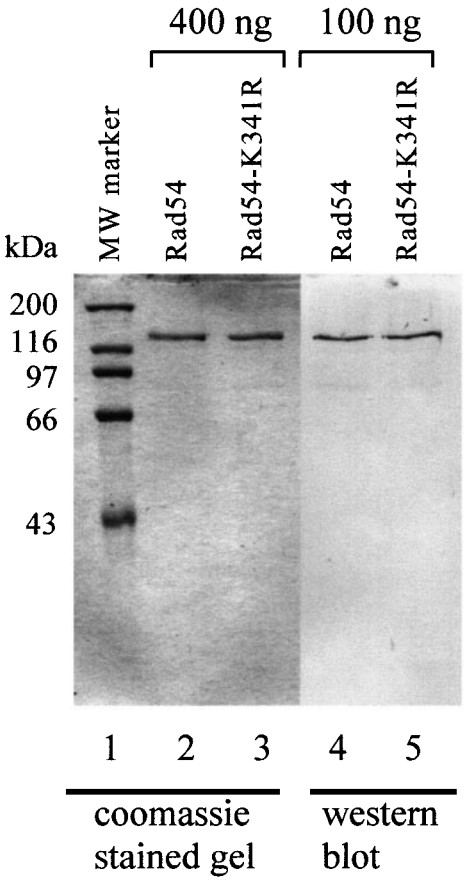

Figure 2. Purification of Rad54 protein. Purified Rad54 and Rad54-K341R were analyzed by SDS-PAGE and visualized with Coomassie brilliant blue R250 (lanes 2 + 3) or Western blot analysis (lanes 4 + 5): lane 1, molecular mass marker (molecular masses are indicated on the left in kDa); lane 2, 400 ng of Rad54 protein; lane 3, 400 ng of Rad54-K341R protein; lane 4, 100 ng of Rad54 protein; lane 5, 100 ng of Rad54-K341R protein. The faint band below GST-Rad54 are probably degradation products, because they are recognized by the specific anti-Rad54 antibodies in the immunoblotting experiment (lanes 4 and 5).

In vitro recombination reactions using *S. cerevisiae* proteins (Figure 4(a)) are typically carried out at 37 °C (New *et al.*, 1998; Shinohara & Ogawa, 1998; Sung, 1994), although the normal growth temperature for yeast is 30 °C (Sherman, 1991). Previous work with differently tagged yeast and human Rad54 proteins suggested the possibility that the Rad54 ATPase activity is unstable at 37 °C (Figure 2(a) of Petukhova *et al.*, 1998; Figure 2(c) of Petukhova *et al.*, 1999; Table 1 of Swagemakers *et al.*, 1998). Before studying the effect of Rad54 protein in *in vitro* recombination reactions, we decided to address this possibility directly by measuring ATPase activity at different incubation temperatures. At 37 °C, significant loss of ATPase

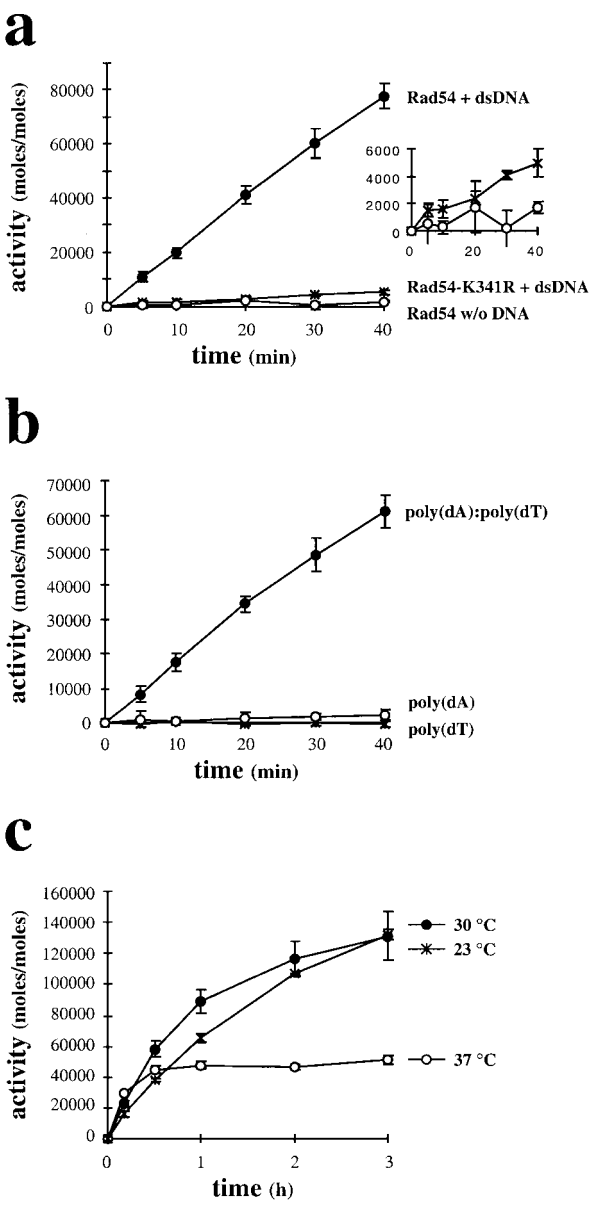

Figure 3. ATPase activity of Rad54 protein. (a) Time course experiments at 30 °C using Rad54 protein with dsDNA (●), Rad54-K341R protein with dsDNA (∗) and Rad54 protein without DNA (○) as cofactor. The inset magnifies the portion of the graph with low ATPase activity. (b) ATPase activity of Rad54 protein is dependent on dsDNA. Time course experiments at 30 °C using Rad54 protein with poly(dA)·poly(dT) dsDNA (●), poly (dA) ssDNA (○) and poly(dT) ssDNA (∗) as cofactors. (c) ATPase activity of Rad54 protein is unstable at 37 °C. Time course experiments with Rad54 protein used ds herring sperm DNA and were carried out at 23 °C (∗), 30 °C (●) and 37 °C (○). All experiments were carried out in triplicate and averages with standard deviations are shown. ATPase activity is expressed as moles ATP hydrolyzed per mol Rad54 protein.

activity was observed after ~30 minutes (Figure 3(c), and data not shown). At 30 °C, only a very gradual loss of ATPase activity was observed in reactions lasting up to three hours (Figure 3(c)). A further decrease in the temperature to 23 °C did not enhance stability of ATPase activity (Figure 3(c)).

Our analysis of the Rad54 ATPase activity agrees with and extends previous studies (Petukhova *et al*., 1998, 1999; Swagemakers *et al*., 1998). Both the dependence of the Rad54 ATPase activity on dsDNA and the functional importance of the ATP-binding motif were established before (Petukhova *et al*., 1999; Swagemakers *et al*., 1998). We find that the Rad54 ATPase activity is unstable at 37 °C. A gradual decline of activity could be seen as soon as five to ten minutes after the start of the reaction (data not shown) leading to a complete loss of activity after 30-60 minutes (Figure 3(c)). This observation is consistent with published results using a different yeast Rad54 fusion protein or a tagged human Rad54 protein (Petukhova *et al*., 1998, 1999; Swagemakers *et al*., 1998). This strongly suggests that Rad54 protein did not lose activity because of the fusion to GST, but that the sensitivity to higher temperatures is an intrinsic property of Rad54 protein itself. The loss of activity was reduced, but still evident, at 30 °C. To avoid problems caused by rapid inactivation of Rad54 protein, all subsequent reactions were carried out at 30 °C.

Rad54 protein stimulates the DNA strand exchange reaction promoted by Rad51/Rpa at 30°C

In vitro recombination reactions using Rad51 protein and Rpa (Figure 4(a)) are typically carried out at 37 °C, and the effect of the yeast Rad54 protein was previously studied under these conditions (Mazin *et al*., 2000a,b; Petukhova *et al*., 1998, 1999). These studies used short incubation times (⩽30 minutes), short DNA substrates, or both, so that the instability of Rad54 protein may not have had an effect. Therefore, we wondered if and how Rad54 protein would stimulate Rad51/Rpa-mediated DNA strand exchange between ΦX174 circular ssDNA and linear dsDNA at 30 °C (Figure 4(a)). DNA strand exchange by Rad51/Rpa at 30 °C showed slower kinetics than at 37 °C (data not shown), but Rad54 protein also stimulated DNA strand exchange at 30 °C (Figure 4(b) and (c)). Storage buffer was routinely added to all reactions lacking Rad54 protein to rule out non-specific effects of the buffer components. Rad54 protein significantly stimulated the initial rate of joint molecule formation. For example, at one hour, the addition of Rad54 protein resulted in a 2.5-fold increase in the yield of joint molecules (Figure 4(c), left). However, Rad54 protein had no effect on the final yield of joint molecules (Figure 4(c)). This stimulation was dependent on the ATPase activity of Rad54, as Rad54-K341R protein did not exhibit

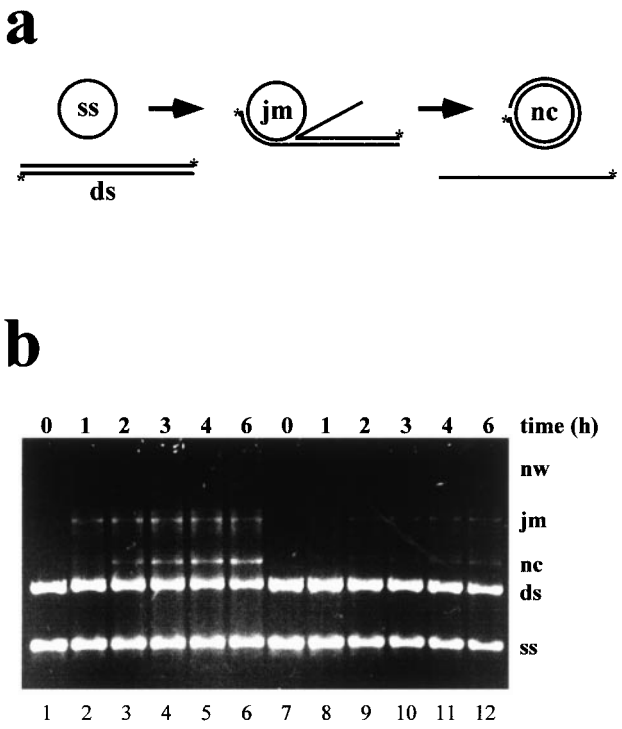

Figure 4. Rad54 protein stimulates DNA strand exchange by Rad51/Rpa at 30 °C. (a) Schematic representation of the DNA strand exchange reaction using ΦX174 circular ssDNA (ss) and *Pst*I-digested linear dsDNA (ds) labeled at the 5′ ends (*). The products of the reaction are joint molecules (jm) and nicked circular DNA (nc). (b) Time course of DNA strand exchange with Rad51 protein and Rpa in the presence of Rad54 protein (left, lanes 1-6) or Rad54-K341R protein (right, lanes 7-12). Ethidium bromide stained bands after electrophoresis on native agarose gels are indicated (nw, networks; jm, joint molecules; nc, nicked circles; ds, dsDNA; ss, ssDNA). Under these experimental conditions (0.2 μM Rad54 protein), only few networks are formed (see Figure 5(b)). (c) Rad54 protein stimulates the formation of joint molecules. The radioactively labeled strands (* in (a)) were quantified using a PhosphoImager. Time course experiments were performed as in (b) and contained Rad54 protein (○), Rad54-K341R protein (∗) or storage buffer (●). The graphs show the formation of joint molecules, nicked circles and total products as indicated. All DNA strand exchange assays were carried out in triplicate and averages with standard deviations are shown.

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype
FF18733	*MATa leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1*[a]
FF18973	*MATa leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1 rad54-Δ::LEU2*[a]
WDHY668	*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1-Δ1.6R can1*
	MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1-Δ1.6R can1
WDHY669	*MATα leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1*
	MATa leu2-3,-112 ura3-52 his7-1 lys2-1
WDHY947	*MATα leu2-3,-112 lys2-1 his7-1 ura3-52 rad54-Δ::URA3*
WDHY949	*MATa leu2-3,-112 trp1-289 lys1-1 his7-2 ura3-52 rad54-Δ::URA3*
WDHY1202	*MATα leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1*
	MATa leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1
WDHY1203	*MATα leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1 rad54-Δ::LEU2*
	MATa leu2-3,-112 trp1-289 ura3-52 his7-2 lys1-1 rad54-Δ::LEU2
WDHY1272	*MATa leu2-3,-112 lys1-1 his7-2 trp1-289 ura3-52 GST-RAD54*
WDHY1273	*MATα leu2-3,-112 lys2-1 his7-1 trp1-289 ura3-52 GST-RAD54*

[a] Kindly provided by F. Fabre.

this effect. In contrast to the stimulation of joint molecule formation, no significant increase in either the rate of formation or the final yield of nicked circles was observed in reactions containing wild-type Rad54 protein (Figure 4(c)). Previous studies examining the effect of Rad54 protein in Rad51 protein-mediated DNA strand exchange could not easily analyze the effect on joint molecule or nicked circle formation, as Rad54 protein stimulated the formation of high molecular mass networks that did not enter the gel (Petukhova *et al.*, 1998, 1999). The formation of such networks is variable and highly dependent on the specific reaction conditions in RecA-mediated DNA strand exchange, and the phage T4 uvsX protein will catalyze network formation under all conditions examined (Bianco *et al.*, 1998). Under our condition, less networks are formed which allows the quantitation of joint molecules and nicked circle formation (see Figure 5). This difference to the previous studies (Petukhova *et al.*, 1998, 1999) may be related to the use of different protein preparations and reaction conditions.

To determine the optimal amount of Rad54 protein needed to stimulate Rad51/Rpa-mediated DNA strand exchange, we titrated the reaction with Rad54 protein (Figure 5). Stimulation of joint molecule formation showed a broad optimum between 0.2 and 0.6 μM Rad54 protein. Little stimulation of nicked circle formation was seen, consistent with the time course experiments (Figure 4). The optimum for stimulating formation of high molecular mass networks could not be reached. Technical reasons did not allow us to add more Rad54 protein to the reactions. Joint molecule formation was inhibited at Rad54 concentrations above 0.6 μM, as more high molecular mass networks were formed. The addition of Rad54-K341R protein inhibited the formation of nicked circles even at low concentrations, which is consistent with the results of the time course experiments (Figure 4). We decided to perform all experiments at 0.2 μM of Rad54 protein, the concentration that provided good stimulation of joint molecule formation, while keeping the formation of high molecular mass networks low. This concentration of Rad54 protein and its ratio to Rad51 protein is similar to the Rad54 protein concentration used previously in DNA strand exchange (Petukhova *et al.*, 1998, 1999) but lower than in studies employing the D-loop assay (Mazin *et al.*, 2000a,b; van Komen *et al.*, 2000).

Rad54 protein stimulates hDNA formation

The effect of Rad54 protein in Rad51-mediated DNA strand exchange and D-loop formation has previously been examined in gel assays that do not allow a direct measurement of overall hDNA formation (Mazin *et al.*, 2000a,b; Petukhova *et al.*, 1998, 1999; van Komen *et al.*, 2000). To further characterize the effect of Rad54 protein on Rad51 protein-mediated DNA strand exchange, we decided to measure hDNA formation directly by utilizing the S_1 nuclease assay. This assay has been used extensively in the analysis of the RecA protein (Bianco *et al.*, 1998; Menetski *et al.*, 1990; Rehrauer & Kowalczykowski, 1993). A homogeneously labeled dsDNA was used as a substrate and the addition of the ssDNA-specific S_1 nuclease caused the release of radioactivity corresponding to the amount of hDNA formed (Figure 6(a)). The samples for the S_1 nuclease assay were taken from the same DNA strand exchange reactions shown in Figure 4(c), to allow a direct comparison of the results. Rad54 protein stimulated the formation of hDNA while the mutant Rad54-K341R protein

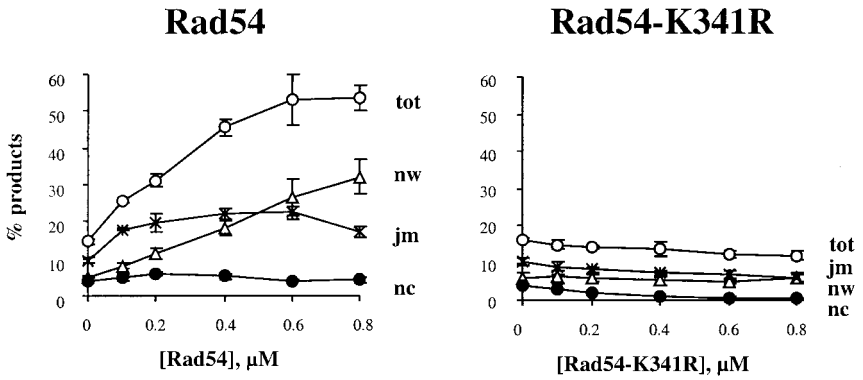

Figure 5. Rad54 protein titration in Rad51-mediated DNA strand exchange. Titration of Rad54 protein (left) and Rad54-K341R protein (right) in DNA strand exchange. Reaction products were measured after two hours. Joint molecule (jm, X), nicked circle (nc, ●), networks (nw, △), and total products (tot, ○) are plotted in dependence of Rad54 concentration. The total amount of storage buffer was the same in all reactions. All experiments were carried out in triplicate and averages with standard deviations are shown. Under standard reaction conditions (±0.2 μM Rad54 protein or Rad54-K341R protein), in the absence of Rad54 protein 5.5 % networks were formed, in the presence of Rad54 protein and Rad54-K341R protein 11.5 % and 5.9 %, respectively.

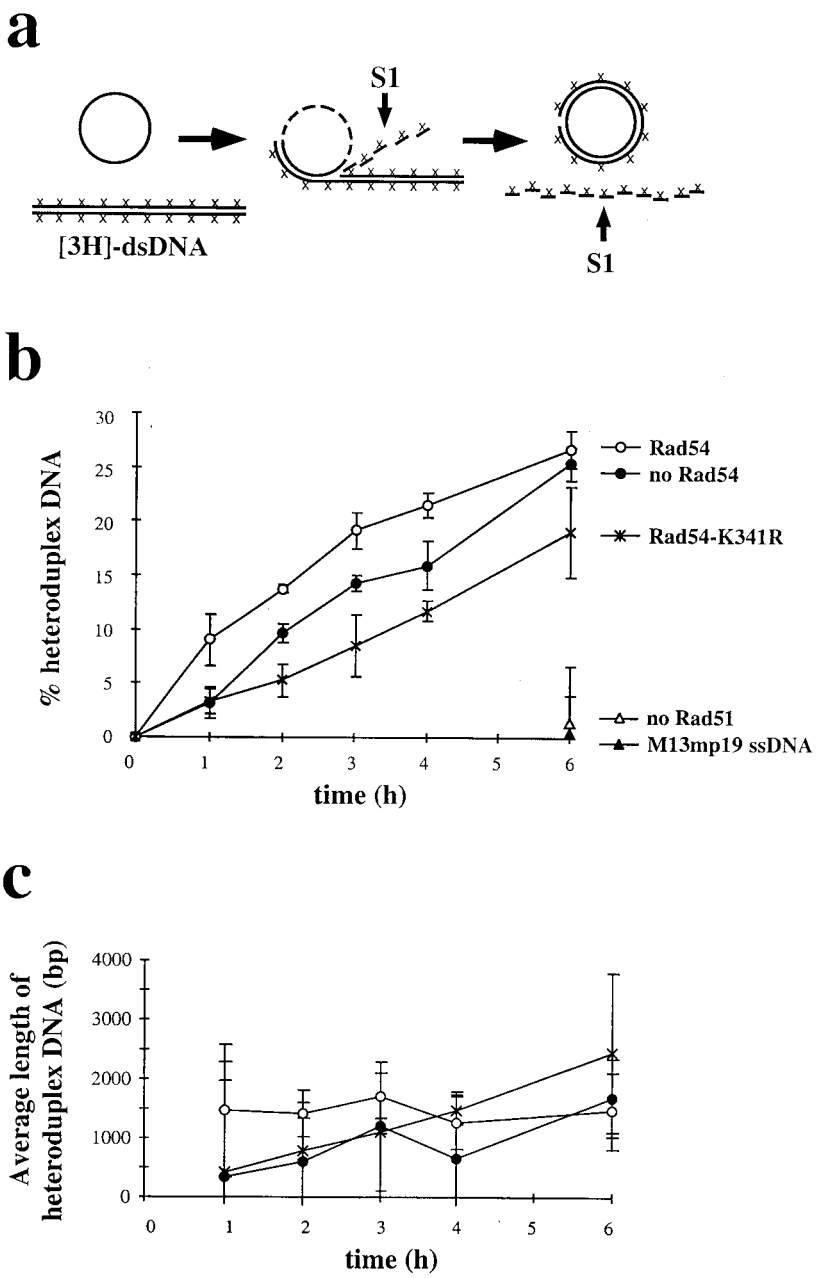

Figure 6. Rad54 protein stimulates the formation of hDNA. (a) Schematic representation of the S_1 nuclease assay. DNA strand exchange reactions as in Figure 4 containing homogeneously ^{3}H-labeled dsDNA (indicated by x) can be digested with S_1 nuclease to yield acid soluble radioactivity that can be readily measured. The length of the digested ssDNA corresponds to the length of hDNA formed. The dsDNA was additionally labeled at its 5′ ends to allow quantification of ongoing DNA strand exchange (see Figure 4). (b) S_1 nuclease reactions containing Rad54 protein (○), Rad54-K341R protein (∗) or storage buffer (●). Negative controls lacking Rad51 protein (but containing Rad54 protein) (△) and with heterologous circular ssDNA form bacteriophage M13mp19 (▲) are included in the graph. All S_1 nuclease assays were carried out in triplicate and averages with standard deviations are shown. (c) The average length of hDNA per joint molecule is not affected by Rad54 protein. The results from the S_1 nuclease assay (b) and the amount of joint molecules in the strand exchange reaction (Figure 4(c)) were used to calculate the average length of hDNA per joint molecule formed. The substrate DNA, ΦX174, has a length of 5386 bp. The nicked circles were assumed to have full length hDNA and were subtracted in this analysis.

exhibited an inhibitory effect (Figure 6(b)). In control reactions, where the Rad51 protein was omitted, no hDNA was formed (Figure 6(b)). This control also showed that Rad54 protein was unable to perform DNA strand exchange or form hDNA on its own. Further controls showed that hDNA formation was entirely dependent on DNA homology: substituting ΦX174 with M13 ssDNA abolished hDNA formation (Figure 6(b)).

hDNA can be formed at two distinct steps in this reaction: by initiation of new joint molecules, resulting in more joint molecules with shorter average length of hDNA; or by elongation of the already pre-existing paired molecules, resulting in joints with longer average hDNA length. To examine which process is stimulated, the average hDNA length of joint molecules was calculated, as described for RecA protein (Menetski *et al.*, 1990; Rehrauer & Kowalczykowski, 1993). The S_1 nuclease assay gives the total amount of hDNA in bp (Figure 6(b)) and the agarose gel assay the percentage of joint molecules (Figure 4(c)) formed in the same DNA strand exchange reaction. By dividing the total base-pairs of hDNA by the number of joint molecules, the average hDNA length per joint molecule was determined (Figure 6(c)). No signifi-

cant differences in the average hDNA length of joint molecules could be observed between the reactions with or without Rad54 protein or with Rad54-K341R protein, as all standard deviations were overlapping (Figure 6(c)). If anything, the presence of Rad54 protein increased rather than decreased the average length of the initial joint molecule (see the one-hour time point in Figure 6(c)). The significance of this is unclear, because the standard deviations are overlapping with those of control reaction without Rad54 or with Rad54-K341R protein. We conclude that Rad54 protein stimulates the formation of joint molecules that exhibit, within the errors of this experiment, the same average hDNA length as in the absence of Rad54 protein.

Rad54 protein interacts with established Rad51 nucleoprotein filaments to stimulate DNA strand exchange

The proper assembly of the protein-DNA complexes needed for DNA strand exchange is critical for the efficient formation of products (New *et al*., 1998; Shinohara & Ogawa, 1998; Sung, 1994, 1997a,b). The optimal assembly is shown in Figure 7(a), top. First, Rad51 protein is allowed to bind to ssDNA and form nucleoprotein filaments. Second, Rpa is added to remove secondary structures in the ssDNA and to facilitate the formation of contiguous presynaptic Rad51-ssDNA filaments (Sugiyama *et al*., 1997). Finally, dsDNA is added to start the reaction.

To establish the step at which Rad54 acts, the protein was added at different times during DNA strand exchange (Figure 7(a)). The addition of Rad54 before Rad51 to the ssDNA was inhibitory (Figure 7(a), *reaction I*). Inhibition was also observed with the mutant Rad54 protein. When Rad54 was added after Rad51, but before Rpa allowed the completion of the filament formation, no inhibition was seen; however, Rad54 protein did not stimulate the reaction under these conditions (Figure 7(a), *reaction II*). Full stimulation of DNA strand exchange by Rad54 protein could only be detected, if it was added after the establishment of presynaptic Rad51-ssDNA filaments (Figure 7(a), *reactions III-V*). To exclude that this effect was the result of the instability of Rad54 protein during the incubations, we performed two control experiments (data not shown). First, direct measurement of the ATPase activity during the reactions showed no correlation between the ATPase activity and the effect on DNA strand exchange. The contribution of the Rad51 ATPase is negligible compared to Rad54. Second, reducing all incubation steps to five minutes each prior to the addition of dsDNA produced results similar to those shown in Figure 7(a). Also using this protocol, Rad54 protein optimally stimulated DNA strand exchange only after the formation of the presynaptic filament (data not shown). Thus, the results indicate that Rad54 protein interacts specifically with established Rad51-ssDNA filaments to stimulate DNA strand exchange.

Rad54 protein interacts with dsDNA and changes its topological conformation (Mazin *et al*., 2000a; Petukhova *et al*., 1999; Tan *et al*., 1999; van Komen *et al*., 2000). To test if the interaction of Rad54 with dsDNA stimulates DNA strand exchange, the protein was preincubated with dsDNA and then mixed with already formed presynaptic Rad51-ssDNA filaments (Figure 7(b)). No effect on product formation could be detected using either storage buffer, Rad54 or Rad54-K341R protein for the preincubation. This result indicates that an interaction of Rad54 protein with dsDNA alone is not sufficient for stimulation of DNA strand exchange.

Rad54 protein is unable to stimulate DNA strand exchange promoted by *E. coli* RecA

A specific interaction with Rad51 nucleoprotein filaments seems to be important for the stimulation of DNA strand exchange by Rad54 protein (Figure 7(a)). This observation is consistent with the described specific interactions between *S. cerevisiae* Rad54 and Rad51 proteins (Clever *et al*., 1997; Jiang *et al*., 1996; Petukhova *et al*., 1998). To test if a similar interaction could also occur with presynaptic filaments formed by other strand exchange proteins, Rad51 protein was replaced with RecA from *E. coli* in the DNA strand exchange reaction. RecA could promote DNA strand exchange under the conditions used for Rad51 protein (data not shown). The addition of Rad54 or Rad54-K341R protein did not stimulate but inhibited the RecA-mediated reaction slightly (data not shown), indicating that Rad54 protein cannot interact with structures formed by RecA to stimulate DNA strand exchange. This underlines the importance of specific protein-protein interactions between Rad54 and Rad51, consistent with earlier observations (Petukhova *et al*., 1999).

Discussion

Rad54 is an important protein of the *RAD52* group, which performs recombinational repair in *S. cerevisiae* and other eukaryotes (Paques & Haber, 1999). Previous studies in yeast have shown that Rad54 protein physically interacts with the eukaryotic RecA homolog, Rad51 (Clever *et al*., 1997; Jiang *et al*., 1996; Petukhova *et al*., 1999) which forms the central nucleoprotein filament in homology search (Ogawa *et al*., 1993; Sung, 1994) and stimulates *in vitro* recombination catalyzed by Rad51 protein and Rpa (Mazin *et al*., 2000a,b; Petukhova *et al*., 1998, 1999; van Komen *et al*., 2000). Here, we focused on the mechanism of this stimulation and found that Rad54 protein specifically enhances the kinetics of joint molecule production in DNA strand exchange leading to an increase in overall hDNA formation. The increase

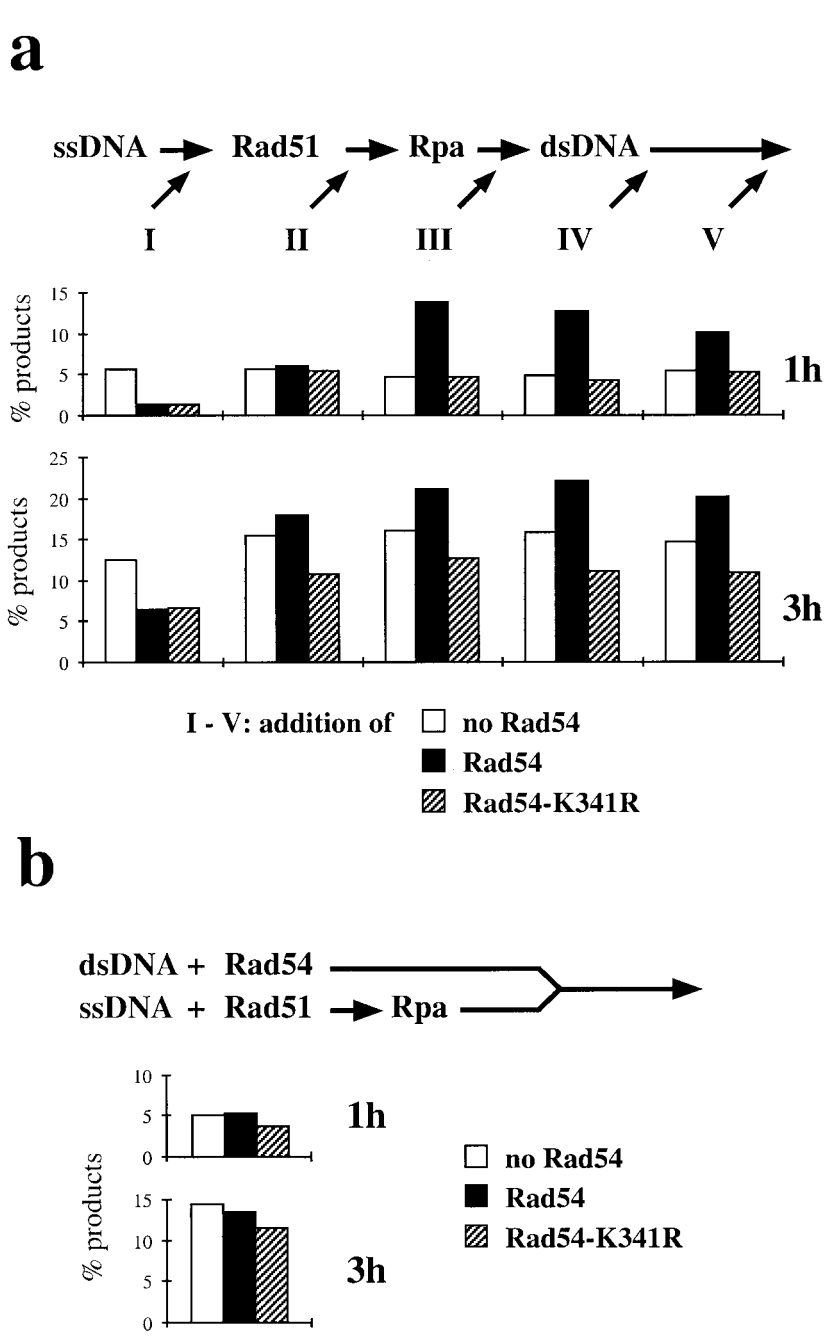

Figure 7. Rad54 protein interacts with established Rad51 filaments to stimulate DNA strand exchange. (a) The order of addition of components in the standard DNA strand exchange reaction is shown in a schematic representation. Rad54 protein (□), Rad54-K341R protein (▨) or storage buffer (■) were added at the indicated time points (I-V) during the reaction and the resulting amount of joint molecules and nicked circles after one and three hours is shown. The detailed incubation times and order of addition was as follows: *reaction I*, ssDNA + Rad54 → 15 minutes + Rad51 → 15 minutes + Rpa → 30 minutes + dsDNA; *reaction II*, ssDNA + Rad51 → 15 minutes + Rad54 → 15 minutes + Rpa → 30 minutes + dsDNA; *reaction III*, ssDNA + Rad51 →15 minutes + Rpa → 25 minutes + Rad54 → 15 minutes + dsDNA; *reaction VI*, ssDNA + Rad51 →15 minutes + Rpa → 30 minutes + dsDNA → 5 minutes + Rad54; *reaction V*, ssDNA +Rad51 →15 minutes + Rpa → 30 minutes + dsDNA → 20 minutes + Rad54; (b) Schematic representation of order of addition variant is shown at the top. Product formation after one and three hours is shown at the bottom. Rad51-ssDNA filament was allowed to form as in standard DNA strand exchange reactions, while the dsDNA was incubated with Rad54 protein (5 μl sample containing buffer for DNA strand exchange) for 15 minutes before addition of the Rad51-ssDNA filament.

in hDNA is explained by the increase in the number of joint molecules. These joints had a similar average hDNA length as those from reactions without Rad54 or with the Rad54-K341R mutant protein. Rad54 protein interacted specifically with the established Rad51-ssDNA filament to facilitate the formation and/or stabilization of the initial homologous joint. We found no effect of Rad54 protein on the formation of the Rad51 nucleoprotein filament. The basis for the ATP-dependent stimulation of Rad51 protein-mediated strand exchange lies in a species-specific interaction between Rad51 and Rad54 proteins, as Rad54 protein had no effect on RecA-mediated strand exchange.

We used two assays to monitor the effect of Rad54 protein in DNA strand exchange catalyzed by Rad51 protein and Rpa. Under our reaction conditions, electrophoretic analysis allowed to separate and quantitate the effect of Rad54 protein on the formation of joint molecule and/or nicked circle products of the reaction (Figure 4), which was not possible in previous studies of Rad54 protein in DNA strand exchange (Petukhova *et al.*, 1998, 1999). In addition, the S_1 nuclease assay allowed us to directly measure the total amount of hDNA pro-

duced (Figure 6(a) and (b)). The S_1 nuclease has been widely applied in studies with RecA protein (Cox & Lehman, 1981) but not yet in studies with Rad51 or Rad54 proteins. Combining the results of the electrophoretic and the S_1 assay gave an estimate of the average hDNA length per joint molecule.

Rad54 protein specifically stimulated the rate of formation, but not the final yield of joint molecules (Figure 4(c)). In addition, the S_1 assay detected an increased rate of hDNA formation (Figure 6(b)). The increased hDNA amounts are mainly due to the formation of larger numbers of joint molecules rather than more extended hDNA in a few joints. Interestingly, one might have expected that formation of more joint molecules would have resulted in a lower average hDNA length per joint. However, this was not observed (Figure 6(c)). Stimulation by Rad54 protein in both assays was dependent on the ATPase activity of Rad54 protein, as the Rad54-K341R mutant protein did not exhibit this activity. The effect of Rad54 protein on joint molecule formation in the Rad51 protein-mediated DNA strand exchange reaction suggests an early role for Rad54 protein in the formation and/or stabilization of joint molecules.

To further define the stage at which Rad54 protein exerts its stimulation, we performed order-of-addition experiments. Optimal DNA strand exchange occurs when the various protein and nucleic acid components are carefully staged. Key is the formation of the Rad51-ssDNA filament which is achieved by sequential addition of Rad51 protein to ssDNA followed by the addition of Rpa, before dsDNA is supplied (New *et al*., 1998; Shinohara & Ogawa, 1998; Sugiyama *et al*., 1997; Sung, 1994, 1997a,b). The results (Figure 7) indicate that ATP-dependent stimulation of DNA strand exchange by Rad54 protein depends on interaction with the established Rad51-ssDNA filament. In addition, preincubation of Rad54 protein with dsDNA did not stimulate DNA strand exchange when mixed with the assembled Rad51-ssDNA filament (Figure 7(b)). These results suggest that Rad54 protein helps the Rad51-ssDNA filament in homology search and joint molecule formation in a coordinated complex and underlines the importance of the prior interaction of Rad54 protein with the filament, before homology search and joint molecule formation. This is consistent with previous observations in the D-loop assay (Mazin *et al*., 2000a,b). The specificity and importance of the Rad51-Rad54 protein interaction is also demonstrated by the species specificity of Rad54 stimulation, as it was not observed in reactions catalyzed by RecA.

Possible model for the role of Rad54 protein in recombinational repair

Several models for the role of Rad54 protein have been suggested. Based on biochemical results it was suggested that Rad54 protein unwinds the dsDNA to allow joint molecule formation by the Rad51-ssDNA filament (Petukhova *et al*., 1999; Tan *et al*., 1999). Using D-loop formation as a model reaction, it was further suggested that the unwinding activity of Rad54 protein on dsDNA is delivered to the pairing site by its interaction with the Rad51-ssDNA filament (Mazin *et al*., 2000a; van Komen *et al*., 2000). Here, we extend that concept by showing that Rad54 protein must interact with the established Rad51-ssDNA filament to stimulate Rad51-mediated homology search, joint molecule formation, and hDNA formation during DNA strand exchange.

Other models invoke the hypothesis that the ATPase activity of Rad54 protein leads to removal of proteins from dsDNA that inhibit recombination, such as nucleosomes (Sugawara *et al*., 1995) or possibly Rad51 protein which is known to inhibit *in vitro* recombination when bound to the target duplex DNA (Bianco *et al*., 1998). The topological change introduced by Rad54 protein may not only stimulate joint formation with naked DNA *in vitro* (Mazin *et al*., 2000a,b; Petukhova *et al*., 1998, 1999; this study), but may also provide further stimulation *in vivo* by inducing the dissociation of nucleosomes (Sugawara *et al*., 1995) or other proteins. The absence of stable joint molecules during DSB repair in *rad54* mutants suggests that Rad54 protein is essential for stable joint formation *in vivo* (Sugawara *et al*., 1995).

The direct interaction of Rad51 and Rad54 proteins in yeast and mammals (Clever *et al*., 1997; Golub *et al*., 1998; Jiang *et al*., 1996; Petukhova *et al*., 1998; Tan *et al*., 1999) provides a physical basis for the specific interaction of Rad54 protein with the presynaptic Rad51-ssDNA filament. A possible model (Mazin *et al*., 2000a; van Komen *et al*., 2000; this study) suggests that Rad54 protein acts after the critical formation of the Rad51-ssDNA filament, which is consistent with results from genetic and cytological studies that suggest a role of Rad54 protein in recombinational repair after that of Rad51 protein. Genetic studies have shown that deletion of the *SRS2* gene is lethal in a *rad54*Δ background (Palladino & Klein, 1992). Srs2 is a DNA helicase (Rong & Klein, 1993) involved in the metabolism of ssDNA gaps, preparing the substrate for the *RAD6* post-replicational repair pathway (Schiestl *et al*., 1990). In the absence of Srs2 protein, these gaps are believed to be channeled into a recombinational repair pathway (Chanet *et al*., 1996). Importantly, mutations in *RAD52*, *RAD51*, *RAD55* and *RAD57*, genes that encode proteins acting in the presynaptic and synaptic phase of recombination (New *et al*., 1998; Shinohara & Ogawa, 1998; Sung, 1997a,b), suppress this synthetic lethality (Schild, 1995). This suggests that the lethality is dependent on attempted recombination and implies that Rad54 protein acts after the formation of the Rad51-ssDNA filament. Results from cytological studies of Rad51 protein foci in prophase of yeast meiosis, which are believed to represent recombination

nodules (Bishop, 1995), provide additional evidence for a role of Rad54 protein following that of Rad51 protein (Shinohara *et al*., 1997, 2000). The formation of Rad51 foci is independent of Rad54 protein, but Rad51 foci fail to turn over in the absence of Rad54 protein (Shinohara *et al*., 2000). This suggests independently that Rad54 acts after Rad51 protein in the recombinational repair pathway.

The experiments presented here and elsewhere demonstrate a synaptic role of Rad54 protein during D-loop formation (Mazin *et al*., 2000a; van Komen *et al*., 2000) and DNA strand exchange (this study), but do not exclude an additional, postsynaptic role for this protein.

Materials and Methods

Strains and plasmids

Standard media and methods for growing and constructing *S. cerevisiae* strains were used. The plasmid pFL39-GAL1-HO containing the HO endonuclease gene under the control of the inducible *GAL1* promoter was kindly provided by P. Linder. Plasmid pWDH424 was constructed by introducing an *Xho*I/*Nhe*I fragment from pWDH195 (Kanaar *et al*., 1996) containing the *RAD54* gene into the *Bam*HI site of the pJN58 vector (kindly provided by J. Nelson) with the *GST* coding sequence under the control of the GAL1 promoter. All ends were made blunt by fill-in reactions with Klenow fragment. The genotypes of all *S. cerevisiae* strains used in this study are indicated in Table 1.

Assays for GST-Rad54 protein function *in vivo*

For growth and survival tests, overnight cultures were diluted to an A_{600} of 1.0, and 4 µl of serial dilutions of 10^0, 10^{-1}, 10^{-2} and 10^{-3} were spotted on plates with or without MMS or irradiated with UV in a Stratalinker. Photographs were taken after two to four days incubation at 30 °C. The test for repair of a DSB caused by the HO endonuclease was performed as follows: overnight cultures were adjusted to an A_{600} of 3 for wild-type strains or an A_{600} of 2 for *rad54*Δ strains. Appropriate dilutions were plated on media containing glucose for titer determination and on media containing galactose for determining survival. Colonies were counted after two days incubation at 30 °C. To generate a chromosomal *GST-RAD54* fusion gene controlled by the native *RAD54* promoter, we used primer olWDH5 (5′-gggCggACAAAgCTACACTgg homologous to bases −746 to −726 of the *RAD54* promoter) and primer olWDH6 (5′-TCCAATAACCTAgTATAggggACATCAg
TTATAAggAAATATATATgg homologous to bases −23 to −1 of *RAD54* promoter and to the first 25 bases of *GST* including an *Eco*NI restriction site) to amplify the *RAD54* promoter from plasmid pWDH444 (Walmsley *et al*., 1997) by PCR using *Pfu*-polymerase. The fragment was cloned into pBlueScript previously linearized with *Sma*I and sequenced. The resulting plasmid containing the amplified promoter fragment was digested with *Nhe*I/*Eco*NI, to liberate the *RAD54* promoter fragment. The *GAL* promoter of pWDH424 was replaced by exchanging the *Xba*I/*Eco*NI fragment with the *Nhe*I/*Eco*NI fragment consisting of the *RAD54* promoter sequence. This procedure ascertained that the *GST-RAD54* was in the same AUG context as *RAD54* normally is. A *Mlu*I/*Eco*RI fragment, containing the *RAD54* promoter and *GST-RAD54*, was transformed in WDHY947 and WDHY949 (*rad54::URA3*) to transplace the construct into the genome using 5-FOA selection. The strains were verified by Southern blot analysis.

Purification of GST-Rad54 protein, Rad51 protein, and Rpa

Rad51 protein and Rpa were purified as described (New *et al*., 1998; Sugiyama *et al*., 1997). The *S. cerevisiae* strain WDHY668 containing plasmid pWDH424 with the *GST* fused to *RAD54* under the *GAL1* promoter was inoculated in 10 ml SD-ura precultures over night. These precultures were transferred to 500 ml SD-ura cultures and again grown over night at 30 °C. Ten 1-l cultures with basic medium −ura (0.17 % (w/v) yeast nitrogen base, 0.5 % (w/v) ammonium sulfate, 2 % (w/v) sodium lactate, 3 % (v/v) glycerol, 0.87 g/l amino acid mix without uracil) were inoculated to an A_{600} of 0.3. After 16 hours at 30 °C, the cells reached an A_{600} of ≈2.0 and were induced by the addition of 1 % (w/v) galactose. The cultures were induced for six hours and then harvested by centrifugation, resulting in ≈45 g wet weight of cells. For storage, the pellets were frozen in liquid nitrogen and kept at −80 °C until needed; 25 g of cells were used per preparation. All steps were carried out at 4 °C. The cells were transferred to a 60 ml Bead beater chamber (Biospec Products) loaded with 30 g of acid-washed glass beads (0.45 mm diameter) and the chamber was filled with buffer A (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 % glycerol, 10 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF, Fluka), 1 µM pepstatin A, 2 mM benzamidin, 1 µM leupeptin and 10 mM sodium hydrogensulfit) containing 1 M NaCl. Cells were disrupted by seven 30 second pulses at two minute intervals. After the last round of disruption the glass beads were washed with buffer A containing 1 M NaCl to yield 50 ml of extract. The extract was centrifuged in a Ti-45 rotor for 45 minutes at 4 °C and 40,000 rpm. The supernatant (fraction I) was precipitated with ammonium sulfate to obtain a fraction from 30-40 % saturation. For each fractionation, solid ammonium sulfate was added slowly, and the mixture was stirred for 0.5 hour followed by a centrifugation for 20 minutes at 4 °C and 10,000 rpm. The pellet was dissolved in 25 ml PBS buffer (2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)) containing 1 M NaCl (fraction II). Fraction II was applied onto a 10 ml glutathion-Sepharose 4B (Pharmacia) column. The column was washed with PBS containing 1 M NaCl and then eluted with buffer A containing 1 M NaCl and 20 mM glutathion. Fractions containing GST-Rad54 protein were pooled (fraction III) and applied onto a Sephacryl S400 (Pharmacia) column equilibrated with buffer A containing 1 M NaCl at a flow rate of 0.3 ml/minute. Fractions containing GST-Rad54 protein (fraction IV) were dialyzed against storage buffer (20 mM Tris-HCl (pH 7.5), 0.1 mM DTT, 0.2 mM PMSF, 1 M NaCl, 60 % glycerol) and stored in small aliquots at −80 °C (fraction V; 1.6 mg/25 g cells). Fraction V was used for all experiments described here.

ATPase assay

Reactions were performed at 30 °C, if not indicated otherwise, in 50 µl (33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM DTT, 20 mM ATP, 0.25 µCi of [γ-^{32}P]ATP (30 Ci/mmol), 90 µg/ml BSA) with 9.2 µM (ntd) of cofactor (herring sperm dsDNA, poly (dA)·poly(dT) dsDNA, poly(dA) ssDNA or poly(dT) ssDNA as indicated) and 15.6 nM Rad54 protein. The ATP concentration was optimized experimentally, and the turnover number was determined at 10 mM and 20 mM ATP. At specific time points, 500 µl of Norite solution (2 % (w/v) Norite charcoal (Sigma C-5260), 0.25 M HCl, 0.25 mM sodium pyrophosphate, 0.25 mM K_2HPO_4) was added to stop the reaction. Samples were incubated on ice for five minutes, then centrifuged for five minutes at 4 °C; 400 µl of supernatant were transferred to 4 ml of scintillation counting cocktail and analyzed in a Beckman scintillation counter.

DNA strand exchange assay

Reactions (10.5 µl) contained 30 mM Tris-acetate (pH 7.5), 1 mM DTT, 50 µg/ml BSA, 20 mM ATP, 20 mM MgOAc, 4 mM spermidine, an ATP-regenerating system consisting of 20 mM creatine phosphate and 1.2 µg creatine kinase, 33 µM (ntd) ΦX174 ssDNA and 10.3 µM Rad51 protein. After 15 minutes incubation at 30 °C 1.8 µM Rpa was added and the reaction was kept at 30 °C for 30 minutes. The time course was started by the addition of 33 µM (nt) ΦX174 dsDNA linearized with *Pst*I and labeled at the 5′ ends using T4 polynucleotide kinase (New England Biolabs), 4 mM spermidine, and either storage buffer, Rad54 or Rad54-K341R protein (0.2 µM, if not indicated otherwise), respectively. The final salt concentration was 30 mM NaCl. The reaction conditions were derived from previous work with Rad51 protein (see Sugiyama *et al*., 1997; Sung & Robberson, 1995). The amounts of spermidine and ATP/MgOAc were optimized by titration. High amounts of ATP were previously shown to prevent aggregate formation in DNA strand exchange with RecA protein (Stasiak & Egelman, 1988). Samples (5.5 µl) were taken at different time points and deproteinized by incubation with 1 µl of 20 mg/ml proteinase K and 1 µl of 10 % (w/v) SDS at 30 °C for 20 minutes. Native gel electrophoresis was performed on 0.8 % (w/v) agarose gels; DNA was subsequently stained with ethidium bromide and the gels were dried for exposure to PhosphoImager screens. All quantifications were performed using ImageQuant software.

S_1 nuclease assay

*Pst*I-digested ΦX174 dsDNA was homogeneously labeled using *Sss*I methylase (New England Biolabs) as described (Renbaum *et al*., 1990). Strand exchange assays were carried out as described above. A part of the reactions (8 µl) was used for the S_1 nuclease assay in 150 µl of 30 mM NaOAc (pH 4.5), 1 mM ZnOAc, 5 % glycerol, 1 % SDS containing 0.4 unit/µl of S_1 nuclease. Samples were incubated at 37 °C for 30 minutes and stopped by adding 4 µl of denatured salmon sperm DNA (10 µg/µl) and 150 µl of 20 % trichloroacetic acid and left for five minutes on ice. After centrifugation for ten minutes 150 µl of supernatant was removed and quantified in 4 ml of scintillation counting cocktail.

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