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The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A

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The human Rad51 protein is homologous to the *Escherichia coli* RecA protein and catalyses homologous pairing and strand transfer reactions *in vitro*. Using single-stranded circular and homologous linear duplex DNA, we show that hRad51 forms stable joint molecules by transfer of the 5′ end of the complementary strand of the linear duplex to the ssDNA. The polarity of strand transfer is therefore 3′ to 5′, defined relative to the ssDNA on which hRad51 initiates filament formation. This polarity is opposite to that observed with RecA. Homologous pairing and strand transfer require stoichiometric amounts of hRad51, corresponding to one hRad51 monomer per three nucleotides of ssDNA. Joint molecules are not observed when the protein is present in limiting or excessive amounts. The human ssDNA binding-protein, hRP-A, stimulates hRad51-mediated reactions. Its effect is consistent with a role in the removal of secondary structures from ssDNA, thereby facilitating the formation of continuous Rad51 filaments.

Keywords: DNA repair/genetic recombination/homologous pairing/RecA/strand exchange

Introduction

The *Escherichia coli* RecA protein plays a central role in homologous recombination by catalysing homologous pairing and strand exchange (Kowalczykowski *et al*., 1994; Camerini-Otero and Hsieh, 1995; Roca and Cox, 1997). *In vitro* studies have shown that the 38 kDa protein binds single-stranded (or partially single-stranded) DNA co-operatively and with high affinity, to form helical nucleoprotein filaments in which the DNA is extended and underwound (Stasiak and Egelman, 1988). The stoichiometry of binding corresponds to one RecA monomer per three nucleotides of ssDNA. Under the same conditions, RecA binds duplex DNA with low affinity (McEntee *et al*., 1981). The filaments made by RecA provide the structural framework in which the search of homology takes place, leading to pairing with naked duplex DNA (Howard-Flanders *et al*., 1984). In this structure, two homologous DNA molecules are brought into close proximity and ATP-dependent strand exchange occurs with a 5′ to 3′ polarity relative to the ssDNA (Cox and Lehman, 1981; Kahn *et al*., 1981; West *et al*., 1981, 1982).

The yeast *RAD51* gene encodes a eukaryotic homologue of RecA (Aboussekhra *et al*., 1992; Basile *et al*., 1992; Shinohara *et al*., 1992; Muris *et al*., 1993; Story *et al*., 1993). *RAD51* is a member of the *RAD52* epistasis group, which includes *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11* and *XRS2* (Haynes and Kunz, 1981; Game, 1983; Ivanov *et al*., 1992; Ajimura *et al*., 1993; Bai and Symington, 1996). Mutations in these genes result in a sensitivity to ionizing radiation and methyl methane sulfonate (MMS), and are associated with defects in meiosis (Petes *et al*., 1991). The *rad51*, *rad52* and *rad54* mutants are also defective in spontaneous and radiation-induced mitotic recombination and in mating-type switching (Game, 1983; Resnick, 1987). Observations showing that *rad51* mutants accumulate, but fail to process, double-strand breaks indicate a direct involvement of Rad51 in the formation of heteroduplex DNA (Shinohara *et al*., 1992).

In vitro studies show that the *Saccharomyces cerevisiae* Rad51 protein forms helical nucleoprotein filaments similar to those observed with *E.coli* RecA (Ogawa *et al*., 1993) and promotes homologous pairing and strand transfer reactions (Sung, 1994). The polarity of strand transfer catalysed by *Sc*Rad51 is opposite to that observed with RecA (Sung and Robberson, 1995). Homologues of *RAD51* have been identified in hyperthermophilic and halophilic archaea (Sandler *et al*., 1996), and in higher eukaryotes including lily (Terasawa *et al*., 1995), *Xenopus laevis* (Maeshima *et al*., 1995), chicken (Bezzubova *et al*., 1993), mouse (Morita *et al*., 1993; Shinohara *et al*., 1993) and humans (Shinohara *et al*., 1993; Yoshimura *et al*., 1993). The high degree of homology found between these proteins suggests that certain aspects of the recombination process have been conserved throughout evolution.

The mammalian Rad51 protein is expressed at high levels in ovarian, testicular and lymphoid tissues, consistent with a role in meiosis and class switch recombination (Shinohara *et al*., 1993). Immunocytochemical studies of mouse and human Rad51 in spermatocytes show that the protein accumulates in discrete foci during meiotic prophase I at the time when chromosomes undergo synapsis (Haaf *et al*., 1995; Ikeya *et al*., 1996; Plug *et al*., 1996; Barlow *et al*., 1997). In mouse somatic cells, Rad51 is induced following treatment with DNA-damaging agents (Haaf *et al*., 1995). High Rad51 expression levels have also been reported in murine B-cells undergoing class switch recombination (Li *et al*., 1996). Taken together, these studies suggest a role for mammalian Rad51 in homologous recombination and recombinational repair. However, it was shown recently that targeted disruption of the murine *RAD51* gene leads to an embryonic lethal phenotype (Lim and Hasty, 1996; Tsuzuki *et al*., 1996), and attempts to generate *rad51*$^{-/-}$ embryonic stem cells have met with failure. These results are surprising in view of the viability of yeast *rad51* mutants and suggest an

essential role for mammalian Rad51 in cell proliferation and/or genome maintenance. Whether this involves interactions with tumour suppressor proteins such as p53 (Stürzbecher *et al*., 1996), Brca1 (Scully *et al*., 1997) and Brca2 (Sharan *et al*., 1997) remains to be determined.

Electron microscopic visualization of hRad51–DNA complexes showed that hRad51 forms nucleoprotein filaments that are similar to those made by RecA (Benson *et al*., 1994). Unlike RecA, however, the protein exhibits similar affinities for single- and double-stranded DNA (Benson *et al*., 1994; Baumann *et al*., 1996). Using single-stranded circular and homologous linear duplex DNA, hRad51 was shown to catalyse ATP-dependent homologous pairing and strand transfer reactions (Baumann *et al*., 1996). Similar results were obtained with oligonucleotide substrates (Gupta *et al*., 1997). To dissect further the role of hRad51 in recombination and repair, the strand transfer reaction has been analysed in detail. We find that hRad51 catalyses ATP-dependent strand transfer with a 3′ to 5′ polarity. We also demonstrate that the human replication protein A (hRP-A), which is known to be involved in DNA replication and repair (Wold, 1997), stimulates joint molecule formation and strand transfer by hRad51.

Results

Polarity of strand transfer

To determine the polarity of strand transfer by the human Rad51 protein, two experiments were performed. In the first, hRad51 was pre-incubated with single-stranded circular (+ strand) DNA to form nucleoprotein filaments, to which we added chimeric ^{32}P-labelled linear duplex DNA that was homologous at either the 5′- or 3′-terminus of the complementary (–) strand (Figure 1A). Following incubation, the products were deproteinized and analysed by agarose gel electrophoresis in the presence of ethidium bromide (which stabilizes joint molecules). Joint molecule formation was observed when the 5′-terminus of the complementary strand was homologous to the ssDNA (Figure 1B, lane b). Addition of hRP-A stimulated the reaction 3-fold and, in its presence, ~ 60% of the DNA formed joint molecules (lane c). In contrast, few, if any, joint molecules were observed when the chimeric linear DNA was homologous at the 3′-end of the complementary strand (lane e). Little stimulation was observed when RP-A was included (lane f). These results indicate that hRad51 exhibits a specificity for the 5′-end of the complementary strand of linear duplex DNA during joint molecule formation.

To determine whether end preference involves hRad51-mediated strand transfer, the second polarity experiment was performed. In this case, hRad51–ssDNA nucleoprotein filaments were reacted with homologous ^{32}P-labelled linear duplexes containing site-specific nicks positioned 174 nucleotides from either the 3′- or 5′-terminus of the complementary (–) strand (Figure 2). When the nick was placed near the 5′-end (*Bam*HI-nicked substrate), efficient and rapid strand transfer was observed (Figure 2, lanes a–g). In contrast, the 3′-terminal fragment (*Nco*I-nicked substrate) was transferred with much reduced efficiency (lanes h–n). Since the polarity of strand transfer is usually defined relative to the single strand on which RecA/hRad51

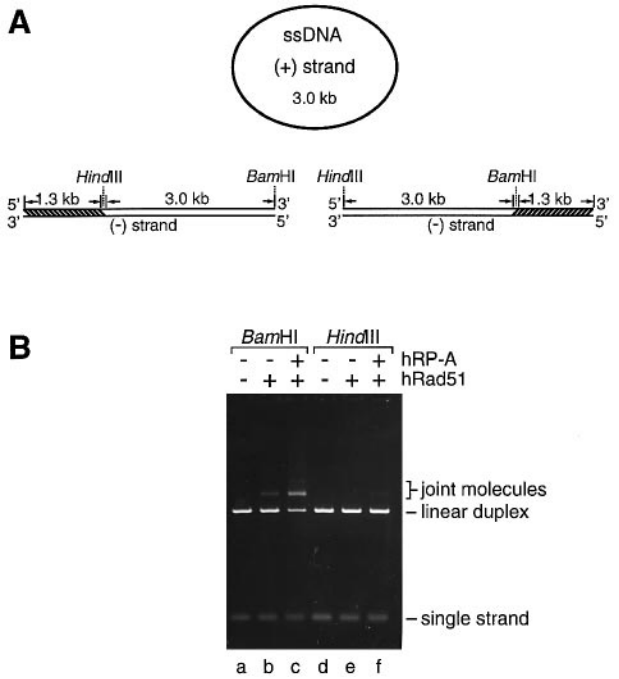

Fig. 1. Joint molecule formation by human Rad51 protein. Reactions contained single-stranded pDEA-7Z DNA, homologous *Bam*HI- or *Hin*dIII-linearized pPB4.3 duplex DNA, hRad51 and hRPA, as indicated. (**A**) Schematic of the DNA species used in this experiment. The striated part of the linear duplex DNA (1.3 kb) is heterologous to the single-stranded circular DNA. (**B**) Ethidium bromide-stained agarose gel of reactions containing *Bam*HI-linearized pPB4.3 (lanes a–c) or *Hin*dIII-linearized pPB4.3 (lanes d–f)

assembles, these results show that hRad51 promotes strand transfer with a 3′–5′ polarity.

Stoichiometric requirement for hRad51

The stoichiometric requirement for hRad51 (in the absence of hRP-A) was determined in strand transfer reactions between single-stranded circular and homologous 5′-^{32}P-end-labelled nicked linear duplex DNA. Efficient strand transfer was observed over a very narrow concentration range, with maximal efficiency occurring at a ratio of one Rad51 monomer per three nucleotides of ssDNA (Figure 3A, lane g). This binding ratio corresponds to the amount of Rad51 required to fully saturate ssDNA (Benson *et al*., 1994), suggesting that efficient strand transfer occurs when the ssDNA is fully coated by Rad51. To quantify the efficiency of strand transfer, the gel shown in Figure 3A was analysed by phosphorimaging (Figure 3A, lower panel). Taking into account that only 33% of the ^{32}P label present on the linear DNA resided in the transferrable fragment, we estimated that under optimal conditions the efficiency of strand transfer approached 85%. Subsaturating amounts of hRad51 strongly reduced strand transfer. Indeed, heteroduplex products were not detected at a ratio of one Rad51 monomer per six nucleotides, corresponding to 50% subsaturation of the ssDNA (lane a). Similarly, strand transfer was inefficient when hRad51 was present in a 30% excess (lanes i and j).

The reactions described above were set up by pre-incubation of Rad51 with ssDNA, to form nucleoprotein filaments, prior to the addition of duplex DNA. To determine whether inhibition by excess hRad51 was due

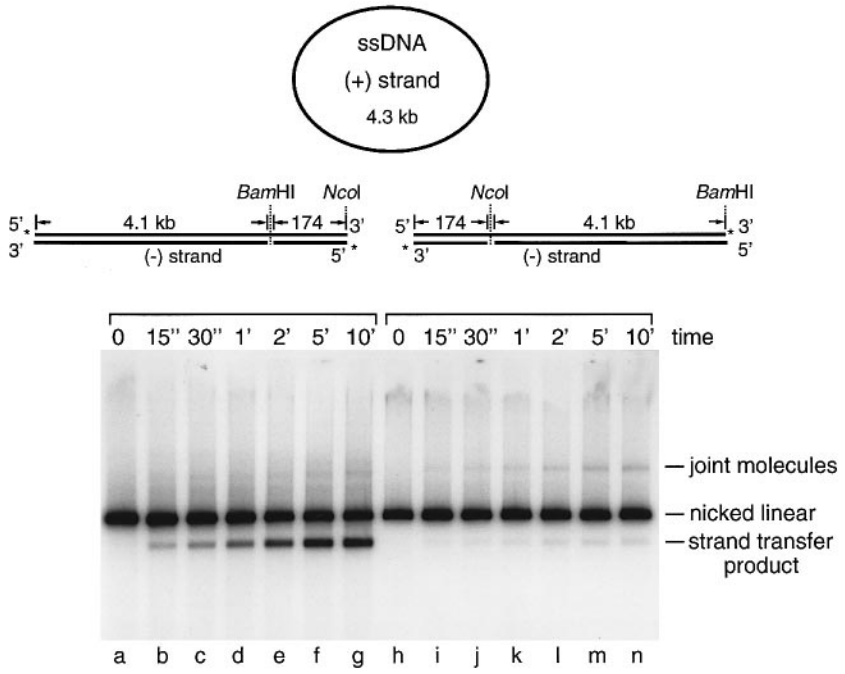

Fig. 2. Polarity of hRad51-mediated strand transfer. hRad51 was incubated with pPB4.3 ssDNA at 37°C for 5 min before the addition of homologous ^{32}P-labelled *Nco*I- or *Bam*HI-linearized duplex DNA containing a site-specific nick as indicated on the diagram. Lanes a–g: *Nco*I-linearized duplex DNA carrying a nick at the *Bam*HI site. Lanes h–n: *Bam*HI-linearized duplex DNA carrying a nick at the *Nco*I site. Reactions were carried out as described in Materials and methods.

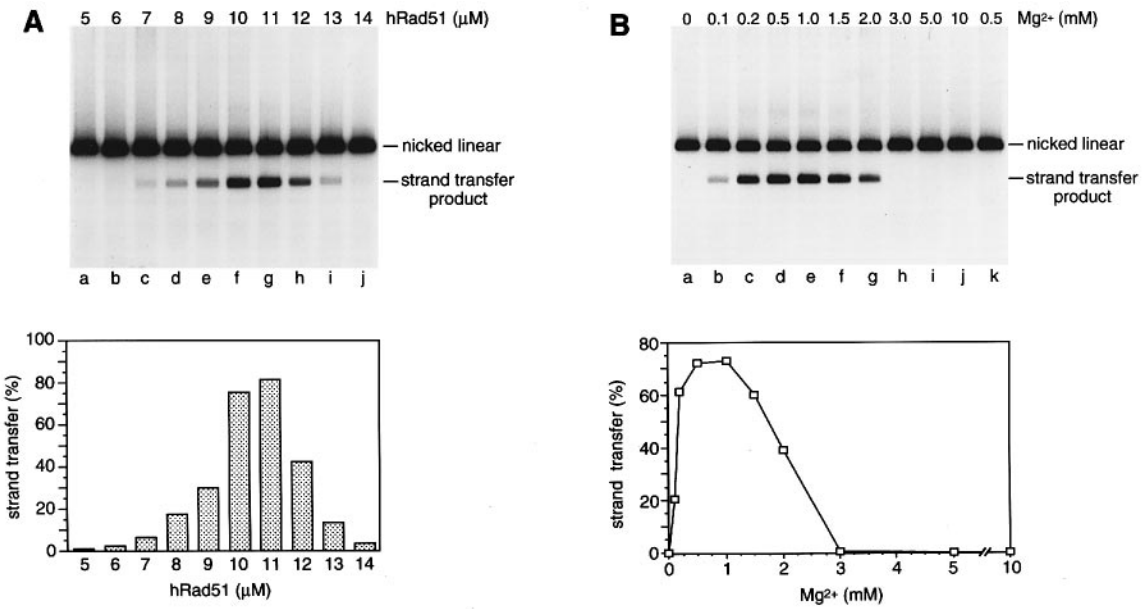

Fig. 3. Strand transfer catalysed by hRad51. (**A**) Stoichiometric requirement for hRad51. Reactions contained single-stranded pDEA-7Z DNA, *Bsa*I-linearized nicked duplex pDEA-7Z DNA and the indicated amounts of hRad51. Incubation and product analysis by gel electrophoresis were as described in Materials and methods. (**B**) Effect of Mg^{2+} concentration on the efficiency of strand transfer. DNAs and hRad51 (10 μM) were incubated as in (A) with the exception that the Mg^{2+} concentration was varied between 0 and 10 mM as indicated. hRad51 was omitted from the reaction of lane k. The strand transfer products were quantified as described in Materials and methods and are shown below the gel.

to its ability to bind duplex DNA, the following experiments were carried out: (i) hRad51 was pre-incubated with nicked duplex DNA prior to the addition of the ssDNA, and (ii) hRad51 was added to a mixture of single- and double-stranded DNA. Strand transfer products were not detected in either experiment (data not shown). These results show that conditions which allow hRad51 to first form filaments on duplex DNA, or permit the equal binding to both single- and double-stranded DNA, inhibit homologous pairing and strand transfer. They lead us to

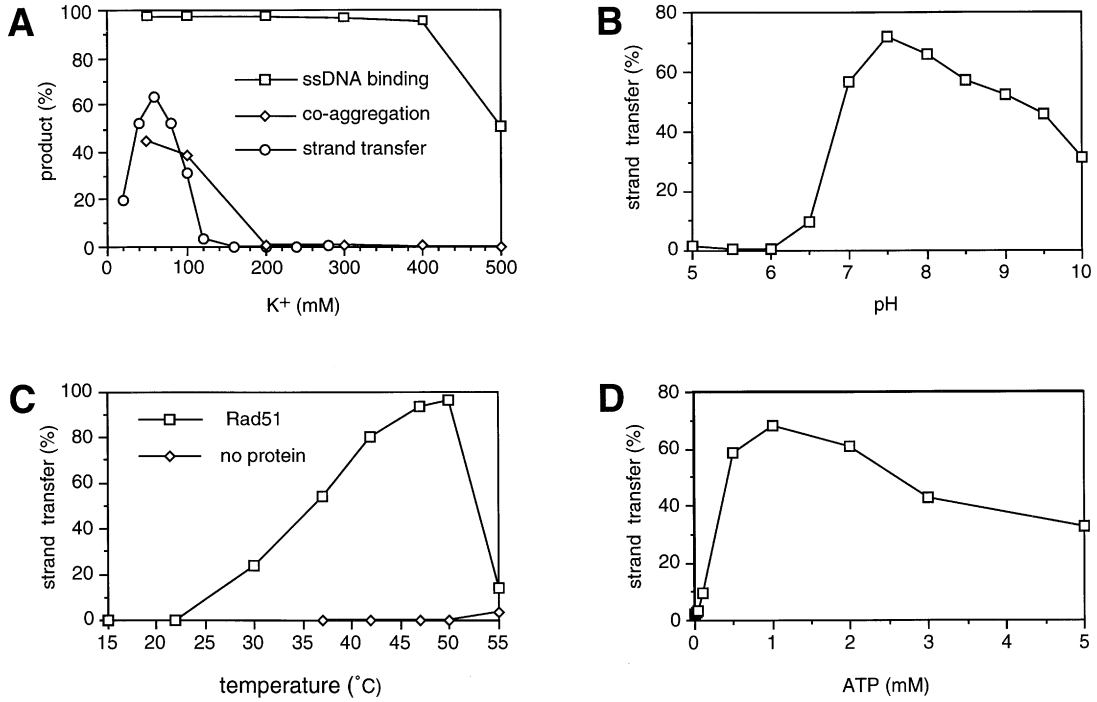

Fig. 4. Co-factor dependence of hRad51-mediated strand transfer activity. Single-stranded pDEA-7Z DNA, ^{32}P-labelled *Bsa*I-linearized homologous duplex DNA carrying a nick at the *Pst*I site and hRad51 were incubated as described in Materials and methods. The standard reaction buffer was adjusted as indicated in each panel. Effect of (**A**) monovalent cations, (**B**) pH, (**C**) temperature and (**D**) ATP concentration, on hRad51-mediated strand transfer. After 30 min, stop buffer was added and all samples were transferred to 37°C for deproteinization prior to analysis by agarose gel electrophoresis. In control samples, hRad51 was replaced by an equivalent amount of protein storage buffer. Panel (A) also shows the effect of monovalent ions on ssDNA binding and co-aggregation, as described in Materials and methods.

suggest that Rad51–ssDNA filaments represent the active structure in the search for homology and subsequent pairing reactions.

Co-factor requirements for hRad51-mediated strand transfer

The requirements for efficient strand transfer are shown in Figures 3B and 4. Optimal activity was observed at 0.2–1.5 mM Mg^{2+} (Figure 3B, lanes c–f). Human Rad51 did not catalyse strand transfer in the absence of Mg^{2+} ions, nor at Mg^{2+} concentrations $\geqslant$3 mM (lanes h–j). Experiments in which hRad51 was loaded onto ssDNA at low Mg^{2+}, followed by a shift up to 10 mM Mg^{2+}, did not produce strand transfer products (data not shown). These results suggest that pairing/strand transfer (rather than filament formation) requires low divalent ion concentrations. Variations in the monovalent ion concentrations revealed optimal activity at 40–80 mM K^+ (Figure 4A). At 20 mM K^+, the efficiency of strand transfer was reduced 3-fold, and the reaction was completely inhibited at >120 mM K^+. Since hRad51 binds ssDNA at K^+ concentrations up to 0.4 M (Figure 4A), the results indicate that inhibition of strand transfer represents an effect on homologous pairing rather than on filament formation. This suggestion was supported by the fact that an essential step preceding homologous pairing, the co-aggregation of the nucleoprotein filament with duplex DNA, was inhibited by 0.2 M KCl (Figure 4A).

Optimal strand transfer was observed at pH 7.5 (Figure 4B), and the efficiency of the reaction increased with temperature between 22 and 50°C (Figure 4C). Nearly 100% strand transfer was observed at 50°C. At this temperature, no spontaneous strand transfer was detected. A further temperature increase to 55°C led to a dramatic drop in the activity of hRad51. The optimal ATP concentration for strand transfer was 0.5–2 mM ATP (Figure 4D).

Stimulation by RP-A

The data presented in Figure 3A showed that the efficiency of strand transfer was dependent upon the accurate titration of hRad51, with small variations in the Rad51/ssDNA ratio leading to a dramatic drop in efficiency. We therefore investigated whether the critical dependence on hRad51 stoichiometry was altered by the presence of the ssDNA-binding protein hRP-A.

ssDNA was incubated with or without 0.26, 0.7 or 1.8 μM hRP-A for 5 min, followed by the addition of hRad51 to final concentrations between 4 and 14 μM. After a further 7 min, during which time hRad51 formed filaments on the ssDNA, ^{32}P-labelled nicked linear duplex DNA was added to initiate the strand transfer reaction. Control reactions showed that in the absence of hRP-A, optimal strand transfer (81%) was observed when the ssDNA was saturated with 10 μM Rad51 (Figure 5A, lane e). At Rad51 concentrations of 8 and 12 μM, the reaction efficiency was reduced to 56 and 45%, respectively (lanes d and f). Below or above these concentrations, few (<6%) strand transfer products were observed (lanes a, b, c and g). However, in the presence of 0.26 μM RP-A (equivalent to one RP-A heterotrimer per 115 nucleotides), the inhibitory effects of elevated hRad51 concentrations were overcome and efficient strand transfer

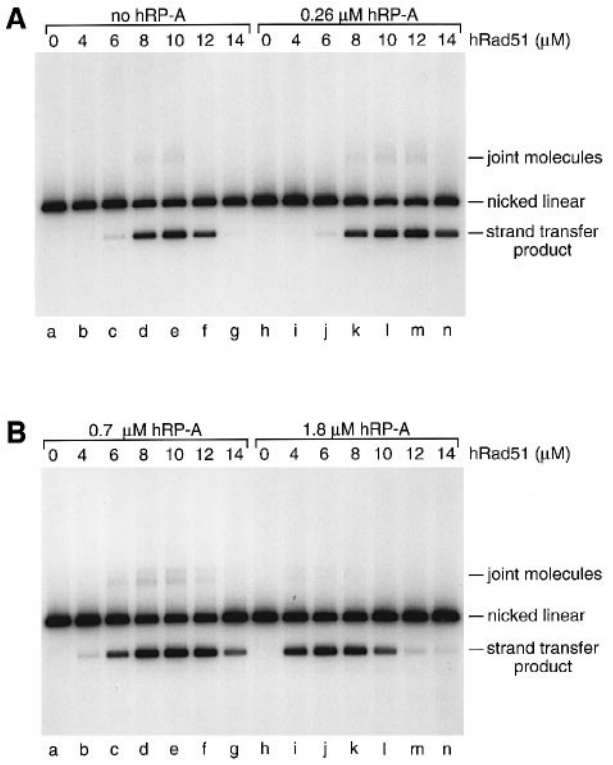

Fig. 5. Effect of hRP-A on hRad51-mediated strand transfer. Reactions containing single-stranded pDEA-7Z DNA, homologous ^{32}P-labelled *Bsa*I-linearized duplex DNA carrying a nick at the *Pst*I site, hRad51 and hRPA were carried out as described in Materials and methods. The hRad51 and hRP-A concentrations were as indicated. DNA products were analysed by agarose gel electrophoresis.

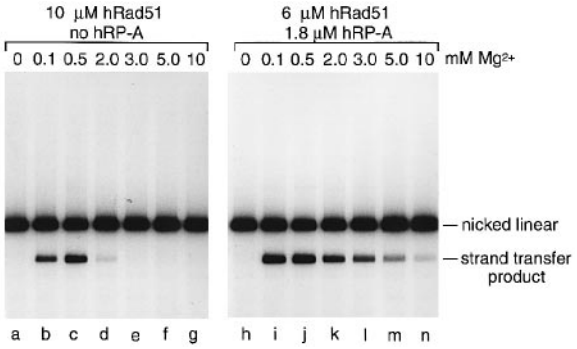

Fig. 6. Effect of hRP-A on Mg^{2+}-dependent hRad51-mediated strand transfer. Single-stranded pDEA-7Z DNA was incubated for 5 min in reaction buffer adjusted to the indicated Mg^{2+} concentrations, without (lanes a–g) or with hRP-A (lanes h–n). hRad51 was then added and strand transfer was analysed as described in Materials and methods.

was observed at 12 and 14 μM Rad51 (Figure 5A, lanes m and n).

At 0.7 μM RP-A (one trimer per 43 nucleotides of ssDNA), efficient strand transfer was observed over a wider range of Rad51 concentrations (Figure 5B, lanes c–g). The width of the hRad51 concentration window was maintained at 1.8 μM RP-A (one trimer per 17 nucleotides); however, the reaction optimum shifted to lower hRad51 concentrations (Figure 5B, lane j). These results show that subsaturating concentrations of hRad51 can promote homologous pairing in the presence of RP-A.

In these experiments, no significant differences were observed whether the ssDNA was pre-incubated with RP-A followed by the addition of hRad51, or vice versa (data not shown). We also found that *E.coli* SSB could substitute for hRP-A, indicating that specific Rad51–RP-A interactions are unnecessary (data not shown).

That Rad51-mediated strand transfer is optimal at low (0.5–2 mM) Mg^{2+} concentrations may in part be due to an inability of hRad51 to remove secondary structures from the ssDNA. Reasoning that RP-A might be expected to remove secondary structures and facilitate hRad51–ssDNA filament formation, reactions were carried out over a range of Mg^{2+} concentrations in the absence (Figure 6, lanes a–g) or presence (lanes h–n) of 1.8 μM hRP-A. The RP-A was found to have a significant stimulatory effect on the ability of hRad51 to promote strand transfer at elevated Mg^{2+} concentrations (compare lanes d–g with k–n).

Discussion

Polarity of strand transfer

Although certain features of the mechanism of homologous recombination appear to have been conserved during evolution (e.g. structural features of the RecA/Rad51 filament), fundamental differences have been observed between *E.coli* RecA and the human Rad51 protein. For example, in reactions between ssDNA and linear dsDNA, RecA forms joints at the 3′- and 5′-ends of the complementary strand of linear duplex DNA (Cox and Lehman, 1981). Subsequent unidirectional strand transfer extends heteroduplex joints formed at the 3′-end, while dissociating those present at 5′-termini (Cox and Lehman, 1981; Kahn *et al*., 1981; West *et al*., 1981, 1982). In contrast, hRad51 exhibits a strong end-preference for joint molecule formation, with heteroduplex joints being detected by gel electrophoresis at 5′-termini only (Figure 1). Similarly, strand transfer by hRad51 initiates from the 5′-end of the complementary linear strand, translating into a 3′–5′ polarity relative to the ssDNA (Figure 2).

With RecA protein, the polarity of strand transfer is related to the structural polarity of the filament relative to the phosphate backbone in ssDNA (Stasiak *et al*., 1988). It is therefore surprising, given the high degree of structural homology between the RecA, yeast and human Rad51 proteins, that the polarity of strand transfer catalysed by RecA (5′–3′ relative to the ssDNA) is opposite to that observed with *Sc*Rad51 (Sung and Robberson, 1995) and hRad51 (this work). These results suggest that the structural polarity of the filament may have inverted during evolutionary processes leading to the development of eukaryotic organisms, possibly in response to the way in which recombination is initiated by double-strand breaks.

DNA binding by hRad51

Using single-stranded and homologous nicked linear duplex DNA, it was shown that efficient strand transfer occurs only over a narrow hRad51 concentration range. Optimal activity occurs at a ratio of one protein monomer per three nucleotides, corresponding to saturation of the ssDNA with hRad51 (Benson *et al*., 1994).

In the presence of ATP, *E.coli* RecA protein shows a much higher affinity for single-stranded, or partially single-stranded DNA, rather than duplex DNA (McEntee *et al*.,

1981). Binding results in the formation of an extended nucleoprotein filament in which the DNA is stretched and underwound (Stasiak and Egelman, 1988). Extended filaments are active with regard to ATPase and strand exchange activity (Yu and Egelman, 1992; Egelman and Stasiak, 1993). Non-extended filaments have been observed in the absence of nucleotide cofactor, or with ADP, but are thought to be inactive (Heuser and Griffith, 1989; Egelman and Stasiak, 1993). In contrast, the *S.cerevisiae* Rad51 protein forms extended filaments on single- and double-stranded DNA in the presence of ATP (Ogawa *et al*., 1993; Sung and Stratton, 1996). Human Rad51 protein also forms filaments on both types of DNA (Benson *et al*., 1994). We have shown, however, that conditions which permit the binding of hRad51 to duplex DNA inhibit strand transfer, indicating that homologous pairing occurs between a hRad51–ssDNA nucleoprotein filament and a naked duplex. This result suggests that filaments formed on ssDNA represent the active complexes necessary for homologous pairing and strand transfer.

The binding of RecA to ssDNA is highly co-operative (Menetski and Kowalczykowski, 1985). When subsaturating amounts of RecA were incubated with DNA, nucleoprotein filaments formed on a fraction of the DNA molecules while others remained uncoated (Stasiak and Egelman, 1988). Those coated by RecA were active for pairing and strand exchange. In contrast to RecA, subsaturating concentrations of hRad51 (one Rad51 per six nucleotides of ssDNA) exhibit an efficiency of strand transfer that is below the limit of detection. These results indicate that the co-operativity of Rad51 binding to ssDNA is significantly lower than that observed with RecA. This proposal is supported by electron microscopy carried out at subsaturating hRad51 concentrations which show partial and segmented coating of all DNA molecules (P.Baumann, F.E.Benson, N.Hajibagheri, A.Stasiak and S.C.West, unpublished observations).

Effect of human RP-A

While it is becoming clear that hRad51 plays a direct role in the search for homology and initial joint molecule formation, recombination is likely to involve a series of factors that act in concert with Rad51. In yeast, Rad51, Rad55 and Rad57 are known to interact (Hays *et al*., 1995; Johnson and Symington, 1995), as are Rad51 and Rad54 (Jiang *et al*., 1996). Biochemical studies on human proteins thought to play a role in recombination indicate interactions between hRad51 and hRad52 (Shen *et al*., 1996b) and between hRad52 and hRP-A (Park *et al*., 1996). Although direct interactions between hRad51 and hRP-A have not been reported, the presence of hRP-A stimulates hRad51-mediated joint molecule formation and strand transfer. In particular, a marked increase in strand transfer activity was observed at sub- and over-saturating hRad51 concentrations and at elevated Mg^{2+} concentrations.

Extensive studies of the actions of ssDNA-binding protein on RecA-mediated strand exchange have shown that it stimulates strand exchange by: (i) facilitating the formation of continuous RecA filaments by removing secondary structures from the ssDNA (Muniyappa *et al*., 1984; Kowalczykowski and Krupp, 1987), and (ii) by binding the released single strand soon after the initiation of strand transfer, thus stabilizing the joint molecules and preventing reinitiation (Chow *et al*., 1988; Lavery and Kowalczykowski, 1992). The role that ssDNA-binding protein plays in facilitating filament formation on ssDNA is thought to be particularly important since RecA does not bind readily to regions of DNA that contain secondary structure. As a consequence, filaments are incomplete. The addition of ssDNA-binding protein greatly facilitates RecA binding such that contiguous stable filaments that coat the entire DNA molecule are formed (Cox *et al*., 1983). Yeast RP-A has also been shown to stimulate filament formation and homologous pairing by *Sc*Rad51 (Sung and Stratton, 1996; Sugiyama *et al*., 1997).

The biochemical studies described here provide evidence of a role for hRP-A in homologous pairing and strand transfer by human Rad51. These results are consistent with hRP-A mediating more efficient filament formation, most likely by removal of secondary structure from the ssDNA. It will now be of interest to extend these studies by inclusion of other proteins such as the human homologues of Rad52 (Muris *et al*., 1994) and Rad54 (Kanaar *et al*., 1996). Moreover, recent studies have identified interactions between Rad51 and several other factors that previously had not been implicated in the process of genetic recombination. These include the tumour suppressor protein p53 (Lim and Hasty, 1996; Stürzbecher *et al*., 1996), the products of the breast cancer genes *BRCA1* (Scully *et al*., 1997) and *BRCA2* (Sharan *et al*., 1997), the ubiquitin-conjugating enzyme Ubc9 (Kovalenko *et al*., 1996), the ubiquitin-like protein Ubl1 (Shen *et al*., 1996a) and a large RNA polymerase II transcription complex (Maldonado *et al*., 1996). Further biochemical studies will help to elucidate the specificity and functionality of these interactions in the context of Rad51-mediated homologous pairing events and the regulation of recombination/repair processes.

Materials and methods

Proteins

Human Rad51 protein was prepared by a method involving spermidine precipitation (Baumann *et al*., 1996, 1997). hRad51 was stored at –70°C in 20 mM Tris–HCl pH 8.0, 200 mM KOAc, 10% glycerol, 1 mM EDTA and 0.5 mM dithiothreitol (DTT). The concentration of hRad51 was determined from its absorption at 280 nm using an extinction coefficient of 1.28×10^4 M^{-1} cm^{-1}. Recombinant human RP-A was produced from the plasmid p11d-tRPA in *E.coli* FB810 and purified by chromatography on Affi-gel blue, hydroxylapatite and MonoQ (Henricksen *et al*., 1994). Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as standard. Restriction enzymes, Klenow fragment of DNA polymerase I and calf intestine phosphatase were purchased from New England Biolabs, T4 polynucleotide kinase was from Pharmacia and BSA from Promega. Protein concentrations are expressed in moles of monomers for hRad51 and moles of trimeric complex for hRP-A.

DNA substrates

The plasmid pDEA-7Z f(+) has been described (Shah *et al*., 1994). Plasmid pPB4.3 f(+) contains a 1.3 kb region of heterology (*Bam*HI–*Hin*dIII PCR fragment derived from *X.laevis* DNA) inserted into the multicloning site of pDEA-7Z f(+). Form I duplex DNA was prepared using Qiagen Plasmid Mega Kits. ssDNA was prepared using the M13 KO7 helper phage following standard protocols.

To generate linear pDEA-7Z f(+) containing a site-specific nick, 180 μg of *Pst*I-linearized duplex DNA was denatured and annealed with 250 μg of pDEA-7Z circular ssDNA in 10 mM Tris–HCl (pH 7.5), 50 mM NaCl and 10 mM $MgCl_2$ by heating to 95°C for 5 min followed by slow cooling to room temperature. Annealed nicked circular dsDNA

was separated from other DNA species using a 1% agarose gel and purified by electro-elution, phenol/chloroform extraction and ethanol precipitation. The DNA was linearized by incubation with *Bsa*I at 43°C for 2 h and dephosphorylated using calf intestine phosphatase (1 U/μg DNA). Finally, the nicked linear duplex DNA was 5′-^{32}P-end labelled using polynucleotide kinase and [γ-^{32}P]ATP.

Nicked linear pPB4.3 DNA was prepared essentially as described above. Form I DNA was digested with either *Nco*I or *Bam*HI, and denatured and annealed with pPB4.3 ssDNA to form nicked circular DNA. Circles containing nicks at the *Bam*HI site were linearized by digestion with *Nco*I, dephosphorylated and then 5′-^{32}P-end labelled using polynucleotide kinase and [γ-^{32}P]ATP. Under the conditions used, little labelling occurred at the nick compared with the two termini. The complementary substrate containing a nick at the *Nco*I site was digested with *Bam*HI and 3′-^{32}P-end labelled with [α-^{32}P]ATP using the Klenow fragment of polymerase I in the presence of 0.1 mM dCTP, dGTP and dTTP. All DNA concentrations are expressed in moles of nucleotides.

DNA binding

The binding of hRad51 (2 μM) to ^{32}P-labelled ssDNA (4.5 μM) was measured as described elsewhere (Baumann *et al*., 1996). Co-aggregation assays (Baumann *et al*., 1996) were carried out using hRad51 (2 μM), ssDNA (12 μM) and ^{32}P-labelled linear duplex DNA (9 μM). The concentrations of monovalent ions were adjusted as indicated in the figure legends.

Homologous pairing assay

Homologous pairing assays (10 μl) between single-stranded circular pDEA-7Z (30 μM) and *Bam*HI- or *Hin*dIII-linearized duplex DNA (pPB4.3; 30 μM) were carried out in 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 100 mM KOAc, 2 mM ATP, 1 mM DTT and 100 μg/ml BSA. hRad51 was mixed with ssDNA on ice, transferred to 37°C for 5 min, at which time hRP-A was added. After a further 5 min, the reaction was supplemented with duplex DNA and incubated at 37°C for 2 h. The final protein concentrations were 10 μM hRad51 and 0.26 μM hRP-A. Reactions were terminated by addition of 2 μl of 5× stop buffer [0.1 M Tris–HCl (pH 7.5), 0.2 M EDTA, 2.5% SDS and 10 mg/ml proteinase K] and the DNA deproteinized by incubation at 37°C for 20 min. The products were analysed on 0.8% agarose gels in TAE buffer run at 5.3 V/cm for 2.5 h, and visualized by ethidium bromide staining. To prevent the dissociation of joint molecules due to spontaneous branch migration, ethidium bromide (1 μg/ml) was also included in the stop buffer, the agarose gel and the gel running buffer.

Strand transfer assay

In standard reactions, single-stranded pDEA-7Z DNA (30 μM) was incubated with hRad51 (10 μM) at 37°C in 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 80 mM KOAc, 2 mM ATP, 1 mM DTT and 100 μg/ml BSA. After 5 min, ^{32}P-end-labelled *Bsa*I-linearized pDEA-7Z DNA (10 μM) containing a nick at the *Pst*I site was added and incubation was continued for 30 min. The final volume was 10 μl. Reactions were stopped and deproteinized as described for the homologous pairing assay.

To determine the polarity of strand transfer, two 80 μl reactions were set up with single-stranded pPB4.3 (30 μM) and hRad51 (10 μM). After 5 min, ^{32}P-labelled nicked linear pPB4.3 DNA (10 μM), containing a site-specific nick close to the 5′ or 3′ end of the complementary strand, was added. Samples (10 μl) were taken at the indicated time points, and the reactions were stopped and deproteinized. DNA products were analysed by electrophoresis through 1.1% agarose gels in TAE buffer run at 5.3 V/cm for 2.5 h. Gels were dried onto filter paper and ^{32}P-labelled DNA was detected by autoradiography and quantified using a Molecular Dynamics Model 425E PhosphorImager.

Variations in protein concentration, buffer composition or reaction conditions are indicated in the figure legends. In reactions where RP-A was added, ssDNA and RP-A were incubated for 5 min at 37°C before the addition of hRad51; followed by a further 7 min incubation and the addition of radiolabelled duplex DNA.

Analysis of the 5′- or 3′-^{32}P-labelled nicked substrates on alkaline gels showed that between 32 and 42% of total radioactivity resides in the small transferable fragment. Variations were observed between the different substrates and between individual labelling experiments using the same substrate DNA. The efficiency of strand transfer is therefore stated as the percentage of radioactivity in the heteroduplex product relative to the maximal transferable radioactivity for each substrate.

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