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 complementarity 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 ScRad51 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 32P-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 32P-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 (BamHI-nicked substrate), efficient and rapid strand transfer was observed (Figure 2, lanes a–g). In contrast, the 3’-terminal fragment (NcoI-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 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’-32P-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 32P% of the 32P 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% saturation 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. 1. Joint molecule formation by human Rad51 protein. Reactions contained single-stranded pDEA-7Z DNA, homologous BamHI- or HindIII-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 homologous to the single-stranded circular DNA. (B) Ethidium bromide-stained agarose gel of reactions containing BamHI-linearized pPB4.3 (lanes a–c) or HindIII-linearized pPB4.3 (lanes d–f).](Image 319x488 to 549x741)
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 32P-labelled NcoI- or BamHI-linearized duplex DNA containing a site-specific nick as indicated on the diagram. Lanes a–g: NcoI-linearized duplex DNA carrying a nick at the BamHI site. Lanes h–n: BamHI-linearized duplex DNA carrying a nick at the NcoI 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, BsaI-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 Mg2+/H11001 concentration on the efficiency of strand transfer. DNAs and hRad51 (10 μM) were incubated as in (A) with the exception that the Mg2+ 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
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 ≥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 c). 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 was restored.
Fig. 6. Effect of hRP-A on Mg\textsuperscript{2+}-dependent hRad51-mediated strand transfer. Single-stranded pDEA-7Z DNA was incubated for 5 min in reaction buffer adjusted to the indicated Mg\textsuperscript{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.

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, *E.coli* RecA forms joints at the 3′- and 5′-ends of the complementary single-stranded pDEA-7Z DNA, homologous BsaI-linearized duplex DNA carrying a nick at the PstI site, hRad51 and hRPA were carried out as described in Materials and methods. 1981. Subsequent unidirectional strand transfer extends the hRad51 and hRP-A concentrations were as indicated. DNA heteroduplex joints formed at the 3′-end, while dissociating products were analysed by agarose 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). At 0.7 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} concentrations (compare lanes d–g with k–n).

**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., 1998).
1981). Binding results in the formation of an extended nucleoprotein filament in which the DNA is stretched and underwound (Stasiak and Egeland, 1988). Extended filaments are active with regard to ATPase and strand exchange activity (Yu and Egeland, 1992; Egeland 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; Egeland 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, nucleo- protein filaments formed on a fraction of the DNA molecules while others remained uncoated (Stasiak and Egeland, 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 (Hayes 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 Mg²⁺ 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 ScRad51 (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 BRCAl (Scully et al., 1997) and BRCAl2 (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 × 10⁴ M⁻¹ cm⁻¹. Recombinant human RP-A was produced from the plasmid p11d-RPA 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 Sigma.

**DNA substrates**

The plasmid pDEA-7Z f(+) has been described (Shah et al., 1994). Plasmid pBP4.3 f(-) contains a 1.3 kb region of heterology (BamHI– HindIII 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 PstI-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 MgCl₂ 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 BsrI at 43°C for 2 h and dephosphorylated using calf intestine phosphatase (1 U/μg DNA). Finally, the nicked linear duplex DNA was 5'-32P-end labelled using polynucleotide kinase and [γ-32P]ATP.

Nicked linear pPB4.3 DNA was prepared essentially as described above. Form 1 DNA wasdigested with either NcoI or BamHI, and denatured and annealed with pPB4.3 ssDNA to form nicked circular DNA. Circles containing nicks at the BamHI site were linearized by digestion with NcoI, dephosphorylated and then 5'-32P-end labelled using polynucleotide kinase and [γ-32P]ATP. Under the conditions used, little labelling occurred at the nick compared with the two termini. The complementary substrate containing a nick at the NcoI site was digested with BamHI and 3'-52P-end labelled with [γ-32P]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 32P-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 32P-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-TZ (30 μM) and BamHI- or HindIII-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 incubated 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-TZ 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, 32P-labelled Bsul-linearized pDEA-TZ DNA (10 μM) containing a nick at the PstI 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 DNA (30 μM) and hRad51 (10 μM). After 5 min, 32P-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 32P-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'-32P-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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References


Human Rad51 protein


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