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The meiosis-specific recombinase hDmc1 forms ring structures and interacts with hRad51

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Eukaryotic cells encode two homologs of *Escherichia coli* RecA protein, Rad51 and Dmc1, which are required for meiotic recombination. Rad51, like *E.coli* RecA, forms helical nucleoprotein filaments that promote joint molecule and heteroduplex DNA formation. Electron microscopy reveals that the human meiosis-specific recombinase Dmc1 forms ring structures that bind single-stranded (ss) and double-stranded (ds) DNA. The protein binds preferentially to ssDNA tails and gaps in duplex DNA. hDmc1–ssDNA complexes exhibit an irregular, often compacted structure, and promote strand-transfer reactions with homologous duplex DNA. hDmc1 binds duplex DNA with reduced affinity to form nucleoprotein complexes. In contrast to helical RecA/Rad51 filaments, however, Dmc1 filaments are composed of a linear array of stacked protein rings. Consistent with the requirement for two recombinases in meiotic recombination, hDmc1 interacts directly with hRad51.

Keywords: hDmc1/hRad51/meiotic recombination/recombinases/ring structures

Introduction

In most eukaryotic organisms, haploid gametes are produced from diploid parental cells by the process of meiosis. The chromosome number is reduced as a single round of DNA replication is followed by two rounds of chromosome segregation: meiosis I and meiosis II (reviewed by Roeder, 1997). Following DNA replication, at the first step of meiotic division (prophase I), a complex series of events occurs including chromosome pairing and synaptonemal complex (SC) formation. During prophase I, the recombination frequency is elevated by 2–3 orders of magnitude. This phenomenon introduces genetic variation and is required for the development of cross-overs, which are usually required for proper chromosome segregation. Defects in meiotic recombination can cause aneuploidy, resulting in abnormal or non-viable progeny.

Our understanding of the process of eukaryotic recombination is built mainly on the phenotypic properties of yeast mutants defective in mitotic or meiotic recombination. These studies indicate that the mechanism of recombination is closely related to the process by which DNA double-strand breaks (DSBs) are repaired. Indeed, meiotic recombination in *Saccharomyces cerevisiae* is initiated at defined sites by the formation of DSBs. At least 10 genes are required for DSB formation in meiotic cells (reviewed by Roeder, 1997; Paques and Haber, 1999). It is thought that DSBs are created by Spo11 protein and that resection involves the *RAD50*, *XRS2* and *MRE11* gene products, giving rise to single-stranded recombinogenic 3′-OH tails. The resected DSBs are then thought to invade homologous duplex DNA, a process that requires the *RAD51*, *RAD52*, *RAD54*, *RAD55* and *RAD57* genes.

The product of *RAD51* (Rad51 protein), a homolog of the bacterial RecA protein, has become the subject of intense interest as a model system to understand recombination in eukaryotic cells. RecA homologs have been found in yeast, hyperthermophilic and halophilic Archaea, *Xenopus*, mouse and humans. The yeast and human Rad51 proteins form helical nucleoprotein filaments on DNA that are similar to those made by RecA, and promote homologous pairing and strand-transfer reactions *in vitro* (reviewed by Baumann and West, 1998).

As expected, the levels of Rad51 are elevated in ovary, testis and lymphoid tissues where mitotic and meiotic recombination events take place (Bezzubova *et al*., 1993; Morita *et al*., 1993). An additional cellular role in DNA metabolism and maintenance of genomic stability is indicated by: (i) the embryonic lethal phenotype of mouse *RAD51*$^{-/-}$ knockouts (Lim and Hasty, 1996; Tsuzuki *et al*., 1996); and (ii) observations showing that repression of a *hRAD51* transgene leads to the accumulation of unrepaired DSBs (Sonoda *et al*., 1998). Consistent with a central cellular role in recombination and repair, hRad51 interacts with a number of key proteins including hRad52 (Shen *et al*., 1996), hRad54 (Golub *et al*., 1997; Tan *et al*., 1999), Xrcc3 (Liu *et al*., 1998), the c-Abl tyrosine kinase (Yuan *et al*., 1998; Chen *et al*., 1999), and the tumor suppressors Brca1 (Scully *et al*., 1997), Brca2 (Sharan *et al*., 1997) and p53 (Stürzbecher *et al*., 1996).

Whereas mitotic recombination is dependent on the functions of the Rad51–57 proteins, meiotic recombination requires, in addition, a second RecA homolog Dmc1 (Bishop *et al*., 1992). The *DMC1* gene, which was originally identified in yeast using a screen for meiosis-specific prophase-induced genes that cause a meiotic defect when disrupted (DMC1, for disrupted meiotic cDNA 1), is required for the synapsis of homologous chromosomes (Bishop *et al*., 1992; Rockmill *et al*., 1995; Yoshida *et al*., 1998). Mutations in *DMC1* lead to defects in reciprocal recombination, the accumulation of resected DSBs, abnormal formation of SCs and cells that arrest at late

meiotic prophase. The mouse homolog of *DMC1* has been cloned (Habu *et al*., 1996) and homozygous *DMC1*$^{-/-}$ knockouts shown to be sterile and to possess smaller than normal reproductive organs (Pittman *et al*., 1998; Yoshida *et al*., 1998). The chromosomes of Dmc1-deficient mouse spermatocytes reveal the presence of unsynapsed homologs.

At the present time, little is known about the mechanism of homologous pairing by Dmc1 protein, a DNA-dependent ATPase that exhibits a weak DNA-pairing activity *in vitro* (Li *et al.*, 1997). Immunocytological studies revealed that Dmc1 and Rad51 show extensive co-localization at multiple sites along yeast meiotic chromosomes (Bishop, 1994). However, while Rad51 is required for the localization of Dmc1, Rad51 focus formation occurs in the absence of Dmc1 (Bishop, 1994; A.Shinohara *et al*., 1997). The appearance of Rad51/Dmc1 foci coincides with the presence of DSBs and the foci tend to disappear as chromosomes synapse. Similarly, homologs of Dmc1 and Rad51 have been shown to assemble into foci during meiosis in lily (Anderson *et al*., 1997) and in mouse spermatocytes (Moens *et al*., 1997). Despite their co-localization, physical associations between Rad51 and Dmc1 have not been demonstrated.

Although genetic studies have provided some information on the roles of Dmc1 and Rad51 in meiotic recombination, the biochemical properties of Dmc1 and potential interactions with Rad51 remain undefined. Here, we show that human Dmc1 protein is a ring protein that can form filament-like structures on double-stranded (ds) DNA. The biochemical properties of hDmc1 are related to those of RecA and hRad51. We also demonstrate that the hRad51 and hDmc1 recombinases interact with each other.

Results

Purification of hDmc1 protein

The *hDMC1* gene was PCR amplified from a human testis cDNA library. Two products were recovered: one encoding full-length *hDMC1* and the second an alternatively spliced form. The full-length product, identical to the published *hDMC1* sequence (Sato *et al*., 1995), was cloned into pET16b to place a His_{10} tag at the N-terminus. Recombinant hDmc1 protein was overexpressed and purified from an *Escherichia coli* strain carrying a deletion of the *recA* gene to avoid possible contamination of hDmc1 with RecA (Figure 1A). Since hDmc1 and hRad51 are >50% identical, the nature of the purified product was confirmed using polyclonal antisera raised against hRad51 (Figure 1B, lane a). A minor degradation product was also detected by Western blotting (lane b). The protein was free of endo- and exonuclease activities, as monitored by the release of acid-soluble counts from ^{32}P-labeled single-stranded (ss) DNA and dsDNA (data not shown).

The native molecular weight of hDmc1 was determined by gel filtration through Superdex 200. hDmc1 eluted with a broad profile, with the bulk of the protein eluting between fractions 24 and 25 (Figure 1C). This protein peak corresponded to a native molecular mass of ~315 kDa, indicative of an octameric form (the calculated molecular weight of the recombinant his-tagged hDmc1 is 40 556 Da). Elsewhere, it has been shown that hDmc1 is indeed octameric (Passy *et al*., 1999b). In addition, a second minor peak was observed in fraction 22, corresponding to a molecular mass of 600 kDa. Monomeric hDmc1 was not detected.

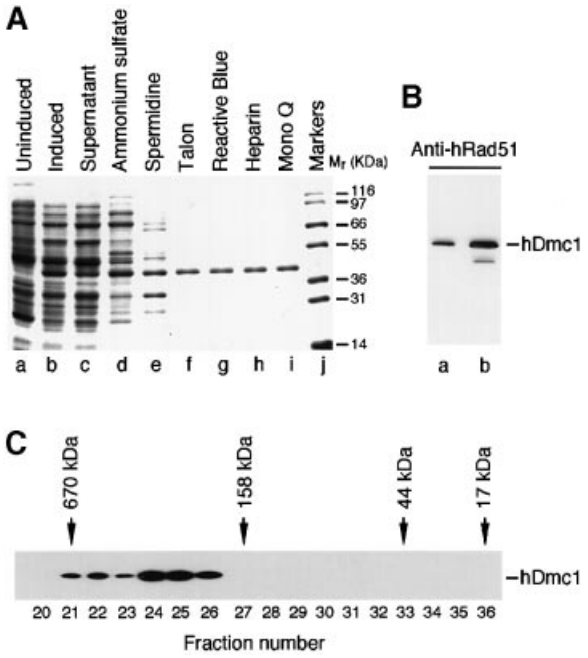

Fig. 1. Purification of hDmc1 protein. (**A**) Overexpression and purification of hDmc1 from *E.coli*. Lanes a and b, cellular proteins before and after induction with IPTG. Lane c, soluble proteins after high-speed spin. Lane d, ammonium sulfate precipitate. Lane e, spermidine precipitate. Lanes f–i, hDmc1 eluted from talon, reactive blue, heparin and MonoQ. Lane j, molecular weight markers. Proteins were separated by SDS–PAGE and visualized with Coomassie Blue. (**B**) Western blot of purified hDmc1 using a pAb (FBE1) raised against hRad51. Lanes a and b contain 50 and 250 ng of purified hDmc1, respectively. (**C**) Multimeric nature of hDmc1. The native molecular mass of hDmc1 was determined by gel filtration through Superdex 200, as described in Materials and methods.

DNA binding and electron microscopic visualization of nucleoprotein complexes

Using electrophoretic mobility shift assays and electron microscopy, the ssDNA- and dsDNA-binding properties of hDmc1 were compared with hRad51. hDmc1 bound both DNAs in a concentration-dependent manner, as seen by the formation of complexes that exhibited reduced mobility through agarose gels (Figure 2A and B, lanes b–f). There was little evidence of cooperative DNA binding. We found that complexes formed by hDmc1 were poorly defined in comparison with those made by hRad51, and tended to smear down the gel.

When hDmc1 was visualized by electron microscopy following negative staining with uranyl acetate (in the presence or absence of DNA), ring-like structures with a diameter of 11–12 nm were observed (Figure 3C). Within each ring a central cavity of ~2–4 nm could be discerned (see the enlargement in Figure 3C, inset). These structures are similar to those observed with RecA (Heuser and Griffith, 1989; Yu and Egelman, 1997) and hRad51 (Baumann *et al*., 1997). A number of doublet rings were also seen (one is indicated by a white arrow) and their presence was consistent with the minor protein peak of twice the mass observed during gel filtration (Figure 1C).

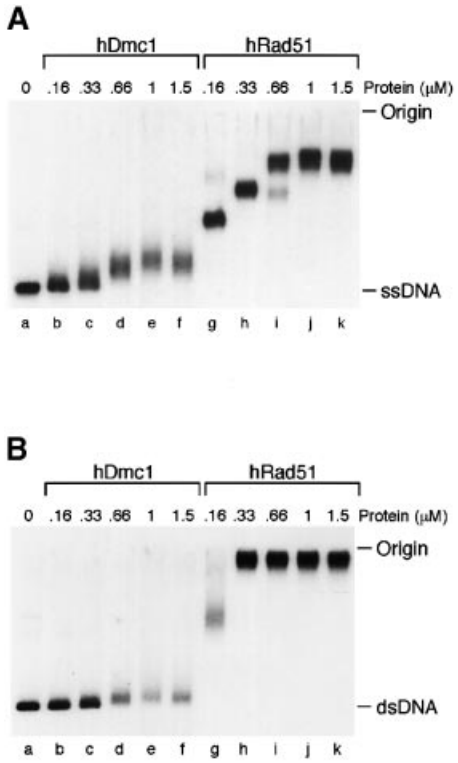

Fig. 2. DNA-binding properties of hDmc1 compared with hRad51. Reactions contained the indicated amounts of hDmc1 (lanes b–f) or hRad51 (lanes g–k) with (**A**) ^{32}P-labeled ssDNA or (**B**) uniformly ^{32}P-labeled nicked circular duplex DNA. Lane a, no protein. Complexes were analyzed by agarose gel electrophoresis.

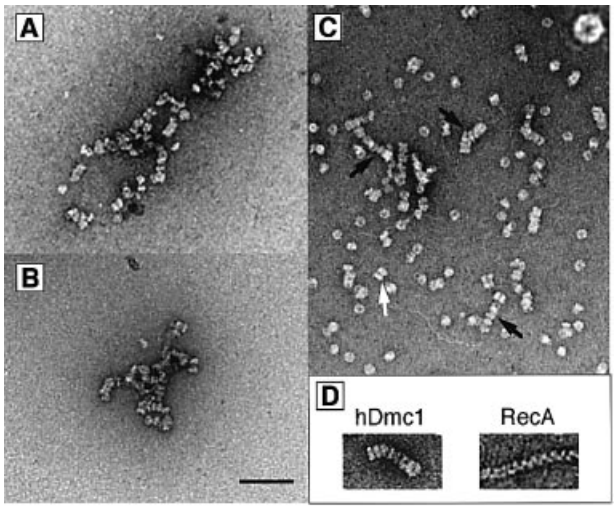

Fig. 3. Electron microscopic visualization of hDmc1–DNA complexes. (**A** and **B**) Electron micrographs of hDmc1–ssDNA complexes. hDmc1 (1.5 μM) was incubated with pJYM4.2 ssDNA (1.5 μM). The bar represents 100 nm. (**C**) Electron micrograph of complexes formed on dsDNA. hDmc1 (1 μM) was incubated with nicked circular ϕX174 dsDNA (3 μM). Doublet rings (white arrow) and filaments consisting of stacked rings (black arrow) are indicated. The insert (top right) indicates an enlargement of a typical hDmc1 ring. (**D**) Close-up view of a hDmc1–dsDNA complex consisting of a series of stacked rings, in comparison with a helical RecA–dsDNA filament. The two images shown were produced at the same magnification.

hDmc1 rings were observed on both ssDNA (Figure 3A and B) and dsDNA (Figure 3C). hDmc1–ssDNA complexes were irregular in structure (Figure 3A) and at higher protein concentrations tended to collapse upon themselves (Figure 3B). In contrast, hDmc1–dsDNA complexes were more clearly defined and rings were seen to be distributed along the DNA. Also apparent were short nucleoprotein filaments consisting of stacked rings (Figure 3C, indicated by black arrows). hDmc1 bound dsDNA with low cooperativity, as seen by the presence of short filaments (containing on average <10 stacked rings) interspersed by regions of naked DNA. The nucleoprotein filaments formed by the stacking of hDmc1 rings were different in structure from the helical nucleoprotein filaments made by *E.coli* RecA (Figure 3D).

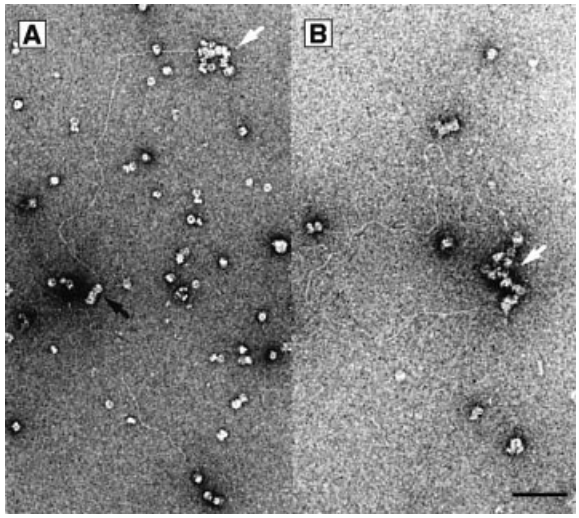

Fig. 4. Electron microscopic visualization of hDmc1 binding the single-stranded regions of tailed or gapped duplex DNA. (**A**) Electron micrograph indicating the binding of hDmc1 (1 μM) to a linear duplex (3 μM) containing a single-stranded tail. The bound ssDNA tail is indicated by the white arrow. A short filament made up of stacked rings on the duplex DNA is indicated by the black arrow. (**B**) Micrograph indicating the binding of hDmc1 (0.74 μM) to gapped circular pPB4.3 plasmid DNA (3 μM). The white arrow indicates the region of ssDNA bound by hDmc1. In this experiment, the hDmc1 was produced from baculovirus-infected insect cells. The bar represents 100 nm.

Since meiotic recombination is thought to be initiated by DNA that contains single-stranded tails, the possibility that hDmc1 may bind specifically to tailed linear duplex molecules was investigated. hDmc1 bound preferentially to the ssDNA tail such that the tail was covered, whereas the duplex DNA remained essentially protein-free (Figure 4A, white arrow). Two further experiments demonstrated specific interaction with ssDNA: (i) binding to ssDNA tails was observed in the presence of a 3-fold excess of linear duplex DNA (data not shown); and (ii) hDmc1 selectively bound the ssDNA regions of gapped circular duplex DNA (Figure 4B, white arrow). At higher concentrations of hDmc1, more extensive covering of the duplex DNA by stacked ring structures was observed (data not shown). Similar results were obtained with His-tagged and untagged hDmc1 protein (data not shown). The preferential interaction of hDmc1 with ssDNA tails may have important biological consequences at the initiation of recombination during meiosis.

Homologous pairing and strand transfer catalyzed by hDmc1

Having established conditions under which hDmc1 interacts with DNA, we next investigated its ability to promote

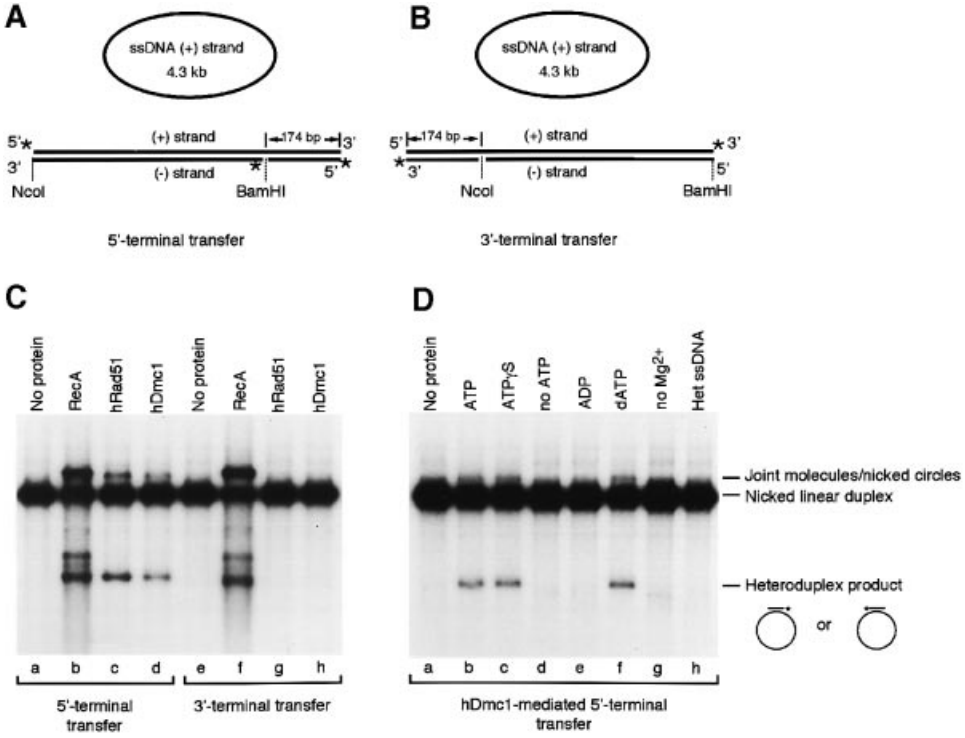

Fig. 5. Strand transfer catalyzed by hDmc1, hRad51 and *E.coli* RecA. (**A** and **B**) Schematic diagram indicating the substrates used to detect transfer of DNA from the 5′ (A) or 3′ (B) end of the complementary (–) strand of ^{32}P-labeled pPB4.3 linear duplex DNA to homologous single-stranded (+) circular DNA. To detect strand transfer, each duplex contains a site-specific nick in the complementary strand permitting the transfer of a 174 nucleotide fragment to the ssDNA. Each asterisk denotes the position of ^{32}P label. (**C**) Comparison of 5′ (lanes a–d) and 3′ (lanes e–h) terminal strand transfer catalyzed by purified RecA, hRad51 and hDmc1 proteins. Strand transfer gives rise to heteroduplex products in which the ssDNA carries a ^{32}P-labeled 174 nucleotide fragment. Reactions were carried out and ^{32}P-labeled products were detected by agarose gel electrophoresis as described in Materials and methods. (**D**) Cofactor dependence of hDmc1-mediated strand transfer. Reactions were carried out in standard buffer using 5 μM hDmc1 (lane b) or in buffer in which ATP was replaced with ATPγS (lane c), ADP (lane e) or dATP (lane f). Lane g, standard buffer lacking Mg^{2+}; lane h, pPB4.3 ssDNA was replaced with heterologous ϕX174 ssDNA; lane a, without protein.

homologous pairing *in vitro*. Using a classical joint molecule assay (circular ssDNA and homologous linear duplex DNA), we were unable to detect the formation of stable joint molecules. We therefore used an alternative assay capable of detecting the formation of stable heteroduplex products. This assay, used previously with hRad51 (Baumann and West, 1997), measures the transfer of a 174-nucleotide-long terminal fragment from nicked linear duplex DNA to homologous ssDNA (as indicated schematically in Figure 5A). We found that hDmc1 promoted homologous pairing and strand transfer, leading to the formation of heteroduplex DNA (Figure 5C, lane d). The efficiency of strand transfer, however, was low in comparison with *E.coli* RecA (lane b) and hRad51 (lane c). Like hRad51 (Baumann and West, 1997), hDmc1 was unable to promote strand transfer when the nick was located close to the 3′-terminus (Figure 5B and C, lanes g and h), indicative of a preferred polarity.

Strand transfer by hDmc1 required the presence of ATP (Figure 5D, lane b), ATPγS (lane c) or dATP (lane f). Strand-transfer products were not observed when ATP was replaced by ADP (lane e), when Mg^{2+} was omitted (lane g) or when heterologous ssDNA was used (lane h).

hDmc1 interacts with the human Rad51 recombinase

Since hDmc1 and hRad51 both promote strand transfer *in vitro*, and their yeast homologs co-localize during meiosis (Bishop, 1994), we looked for genetic and biochemical interactions between the two human recombinases.

First, two-hybrid interactions were analyzed following the transformation of yeast strain pJ69-4A with plasmids bearing full-length hRad51 and hDmc1 fused either to the Gal4 DNA-binding or activation domains (Table I). pJ69-4A was chosen because it contains three reporter genes (*ADE2*, *HIS3* and *lacZ*), each driven by a different promoter (James *et al*., 1996). We found that hDmc1 and hRad51 homotypic interactions gave rise to colonies on omission media after 3 days and a 22- and 12-fold increase in β-galactosidase activity, respectively. This result supports observations indicating that both hRad51 (Baumann *et al*., 1997) and hDmc1 (this manuscript) self-associate. Heterotypic interactions between hRad51 and hDmc1 led to the growth of small colonies after 7 days accompanied by a modest increase in β-galactosidase activity (~3-fold induction).

Secondly, interactions between purified hDmc1 and hRad51 were demonstrated by co-immunoprecipitation. To achieve this, three hDmc1-specific monoclonal antibodies (mAbs 1B2, 1D12 and 4G3) were generated (Figure 6A). Pull-down complexes were analyzed by SDS–PAGE and Western blotting using an antibody raised against hRad51, which also cross-reacts weakly with hDmc1. When mixed at equimolar ratios, hDmc1 and hRad51 co-immunoprecipitated (Figure 6A, lanes g–i). Control experiments

Table I. Two-hybrid interactions between hDmc1and hRad51

Gal4 DNA-binding domain fusion	Gal4 activation domain fusion	Growth on media lacking adenine or histidine	Stimulation of β-galactosidase activity (-fold)
hDmc1	vector	–	1
hRad51	vector	–	1
vector	hDmc1	–	1
vector	hRad51	–	1
hDmc1	hDmc1	++++	22
hRad51	hRad51	++++	12
hDmc1	hRad51	+	2.5
hRad51	hDmc1	+	3

+, growth after 7 days on omission media; ++++, growth after 3 days on omission media.

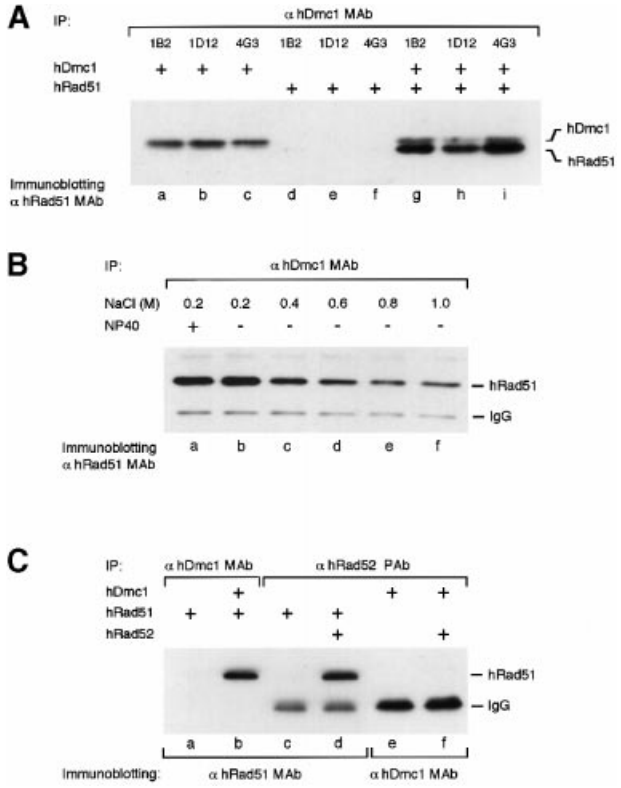

Fig. 6. Co-immunoprecipitation of hRad51 with hDmc1. (**A**) hDmc1, hRad51, or a mixture of both proteins were immunoprecipitated with the anti-hDmc1 mAbs 1B2, 1D12 or 4G3 as indicated, and visualized by Western blotting using an anti-hRad51 mAb. (**B**) Co-immunoprecipitation of hRad51 with hDmc1 from cell-free extracts. Sf9 insect cells were co-infected with *hDMC1* and *hRAD51* baculovirus, and cell-free extracts were prepared. Complexes were immunoprecipitated using the anti-hDmc1 mAb 1D12 and washed with 1% NP-40 containing 0.2 M NaCl (lane a) or in hypotonic buffer containing the indicated amounts of NaCl (lanes b–f). hRad51 was visualized using the anti-hRad51 mAb 14B4. (**C**) Analysis of interactions between hDmc1 and hRad52. Protein mixtures were immunoprecipitated using the anti-hDmc1 mAb 1B2 (lanes a and b), or an anti-hRad52 pAb (lanes c–f), and visualized by Western blotting using the anti-hRad51 mAb (3C10) or an anti-hDmc1 mAb (1D12). Immunoprecipitations with the anti-hRad52 pAb were carried without cross-linking of the antibody to the beads, resulting in an additional band (IgG) on the immunoblot.

showed that the hDmc1 mAbs were specific for hDmc1 (lanes a–c) and failed to cross-react with hRad51 (lanes d–f). The hRad51–hDmc1 complex was resistant to 400 mM potassium acetate and 50 μg/ml ethidium bromide, or DNase I treatment, suggesting that the interactions were not due to the presence of contaminating DNA (data not shown).

Thirdly, extracts from insect cells co-infected with baculovirus carrying *hDMC1* and *hRAD51* were subjected to co-immunoprecipitation with the anti-hDmc1 mAb 1D12. Pull-down complexes were washed extensively with 1% NP-40 detergent (at 0.2 M NaCl) or in buffer containing 0.2–1 M NaCl, and analyzed by Western blotting with a hRad51-specific mAb (14B4). hRad51 interacted with hDmc1 under all conditions tested (Figure 6B).

Because hDmc1 and hRad51 are structurally similar, and hRad51 interacts with hRad52 protein (Shen *et al*., 1996), we also tested whether hDmc1 interacts with hRad52. Polyclonal antibodies raised against hRad52 pulled down a hRad51–hRad52 complex (Figure 6C, lane d) but not hDmc1 (lane f).

Discussion

The recent identification and characterization of DNA repair proteins such as Rad51 and Rad52 has provided our first insight into the mechanisms of the related processes of recombination and DSB repair in eukaryotic organisms. hRad51 has been shown to be structurally and functionally related to the *E.coli* recombinase RecA and, like RecA, promotes interactions between homologous DNA molecules (Benson *et al*., 1994; Baumann *et al*., 1996; Gupta *et al*., 1997). hRad52, which forms ring-like structures *in vitro* (Van Dyck *et al*., 1998), appears to play an early role in the initiation of recombination by binding to resected DSBs (Van Dyck *et al*., 1999) and stimulating joint molecule formation by hRad51 (Benson *et al*., 1998).

The identification of Dmc1, a meiosis-specific homolog of RecA/Rad51, as an essential component of the meiotic recombination machinery in yeast and higher eukaryotes (Bishop *et al*., 1992; Bishop, 1994; Habu *et al*., 1996) now opens the door to biochemical studies of meiosis-specific aspects of the recombination process. As observed with yeast and human Rad51, purified hDmc1 exhibits a weak ATPase activity and promotes ATP-dependent DNA strand-transfer reactions *in vitro* (Li *et al*., 1997). hDmc1 was found to promote heteroduplex formation in the presence of ATP or ATPγS, indicating a requirement for ATP binding rather than extensive ATP hydrolysis. Similar observations have been made with RecA (Menetski *et al*., 1990; Rosselli and Stasiak, 1990) and Rad51 (Baumann *et al*., 1996; Sung and Stratton, 1996).

hDmc1 forms toroidal structures and binds dsDNA to form non-helical nucleoprotein filaments

A remarkable feature of many enzymes involved in DNA metabolism is their toroidal structure (Hingorani and O'Donnell, 1998). Enzymes involved in various steps of recombination, such as the yeast and human Rad52 proteins (Shinohara *et al*., 1998; Van Dyck *et al*., 1998), the *E.coli* branch migration protein RuvB (Stasiak *et al*., 1994), bacteriophage λ exonuclease (Kovall and Matthews, 1997), β protein of bacteriophage λ (Passy *et al.*, 1999a), p22 erf protein (Poteete *et al*., 1983) and *E.coli* RecT protein (Thresher *et al*., 1995), have been shown to form ring structures. This specialized architecture can generate multiple binding sites around the ring or encase DNA within the central cavity. Electron microscopic visualization of hDmc1 revealed that it forms ring structures, with a diameter of ~11–12 nm and a central cavity of 2–4 nm. Elsewhere we present a three-dimensional reconstruction of Dmc1 and show that the rings are composed of eight subunits (Passy *et al*., 1999b).

Although rings of RecA (Heuser and Griffith, 1989) and hRad51 (Baumann *et al*., 1997) have been observed by electron microscopy, the relevance of such structures is presently unclear. Indeed, it is unlikely that they can readily convert into helical nucleoprotein filaments that represent the biologically active form of RecA/Rad51. In the presence of ATP, RecA coats single-stranded or gapped duplex DNA to form filaments in which the DNA is extended to ~1.5× the length of B-form duplex DNA (Stasiak and Egelman, 1988). Similar structures are made by the yeast and human Rad51 proteins (Ogawa *et al*., 1993; Benson *et al*., 1994). In contrast, hDmc1–ssDNA complexes appear as 'beads on a string', rather than as filaments, and at high hDmc1 concentrations the complexes collapse down upon themselves. In accord with data presented elsewhere (Li *et al*., 1997), hDmc1 binds preferentially to ssDNA compared with dsDNA. Preferential binding was particularly evident when the interaction of hDmc1 with tailed linear or gapped duplex DNA was analyzed, in which case hDmc1 was specifically located on the ssDNA regions (Figure 4).

In contrast to ssDNA–hDmc1 complexes, the binding of hDmc1 to dsDNA resulted in the formation of short nucleoprotein filaments. These were interspersed by regions of naked DNA or regions containing a few hDmc1 rings, characteristic of a low cooperativity of binding. Surprisingly, filaments made by hDmc1 were non-helical and were composed of a series of stacked rings. The biological significance of these structures is presently unknown, but they represent the first example of a non-helical filament formed by a member of the RecA/Rad51 class of recombinases and appeared more similar to those made by *E.coli* RecT protein than to those made by RecA (Thresher *et al*., 1995).

DNA strand transfer by hDmc1

In vitro, hDmc1 exhibits a weak strand-transfer activity (Li *et al*., 1997; this work). Using single-stranded circular DNA and nicked linear duplex substrates, transfer of the 5′-terminus of the complementary strand of the linear to the ssDNA was observed. In contrast, transfer of the 3′-terminus was not detected. These results indicate that hDmc1 promotes transfer of the complementary strand with a 5′–3′ preference, similar to that observed with yeast and human Rad51 proteins (Sung and Robberson, 1995; Baumann and West, 1997, 1999). Since the directionality of strand transfer is normally defined relative to the ssDNA on which protein binding occurs, this translates into a 3′–5′ polarity with respect to the ssDNA.

According to current models for the initiation of meiotic recombination, 5′–3′ degradation of the DSB ends results in the formation of duplexes with 3′ssDNA tails that subsequently invade homologous duplex DNA (Paques and Haber, 1999). The specific binding of hDmc1 to tailed duplex DNA (Figure 4A) and the 3′–5′ polarity of Dmc1-mediated strand transfer (Figure 5) are consistent with the initiation of strand transfer from an invading 3′ssDNA tail. *In vivo* data also support the notion that Dmc1 is involved in strand invasion since yeast mutants defective in *DMC1* accumulate abnormally high levels of DSBs and exhibit hyper-resection at these ends (Bishop *et al*., 1992).

Although hDmc1 can form homologous contacts *in vitro*, the efficiency of these reactions is low compared with RecA. This suggests that other factors may be required to increase the efficiency of hDmc1-mediated strand exchange or to promote branch migration once the initial joint has been made. Recently, joint molecule formation by hRad51 was shown to be stimulated by hRad52 (Benson *et al*., 1998). Despite the extensive homology between hRad51 and hDmc1, however, specific interactions between hDmc1 and hRad52 were not detected. Because yeast and human Rad51 proteins interact with Rad54 (Jiang *et al*., 1996; Clever *et al*., 1997; Golub *et al*., 1997; Tan *et al*., 1999) and joint molecule formation by *Sc*Rad51 is stimulated by *Sc*Rad54 (Petukhova *et al*., 1998), it will be interesting to determine whether a recently identified meiosis-specific homolog of Rad54 (Kanaar *et al*., 1996; Klein, 1997; M.Shinohara *et al*., 1997) can modulate Dmc1 activity. Also, it has been shown that the Brca1 and Brca2 proteins, which are implicated in certain forms of breast cancer, interact with hRad51 and co-localize with hRad51 on the axial elements of developing SCs in meiotic cells (Scully *et al*., 1997; Sharan *et al*., 1997; Chen *et al*., 1998). Future studies will determine whether Brca1 and Brca2 interact with hDmc1.

Because Dmc1 and Rad51 proteins are highly homologous and co-localize on meiotic chromosomes (Bishop, 1994; Anderson *et al*., 1997; Moens *et al*., 1997), it is possible that they act in concert or play coordinated roles in meiotic prophase. Indeed, yeast mutants defective in *dmc1* and *rad51* exhibit similar delays in chromosome synapsis and pairing (Weiner and Kleckner, 1994; Rockmill *et al*., 1995). Here we have demonstrated interactions between hDmc1 and hRad51 by (i) two-hybrid analyses, (ii) co-immunoprecipitation of the purified proteins, and (iii) co-immunoprecipitation from cell-free extracts. Two-hybrid interactions between *Sc*Dmc1 and *Sc*Rad51 have not been observed, indicating that the human proteins may differ from their yeast counterparts (Dresser *et al*., 1997).

Although synapsis of homologous chromosomes is independent of recombination in *Drosophila* and *Caenorhabditis elegans*, recombination is a prerequisite for synapsis in budding yeast and mice. Studies of mouse *DMC1*$^{-/-}$ knockouts show that a Dmc1 defect causes sterility due to abnormal spermatocytes and follicles lacking oocytes. In Dmc1-deficient spermatocyte nuclei, chromosomes show

extensive asynapsis despite axial element formation. The biochemical experiments described here contribute to our understanding of the phenotypes of the *DMC1*$^{-/-}$ mouse by suggesting that the absence of Dmc1 results in a recombination defect that leads to abnormal synapsis between homologs and consequently causes sterility.

Materials and methods

Plasmids and DNA

Single- and double-stranded ϕX174 DNA was purchased from New England Biolabs. ssDNA was ^{32}P-labeled as described (Van Dyck *et al*., 1998). pPB4.3 (Baumann and West, 1997) and pJYM4.2 ssDNAs, and pFB585 (Van Dyck *et al*., 1998) and pPB284 (Baumann and West, 1999) dsDNAs were prepared by standard protocols. Nicked circular dsDNA was produced by treatment of form I DNA with DNase I in the presence of ethidium bromide. Nicked linear pPB4.3 was prepared as described (Baumann and West, 1997).

Plasmid pJYM4.2 was constructed as follows. A portion of the *IMP2* gene was PCR amplified from budding yeast genomic DNA using two primers bearing *Eco*RI and *Bam*HI sites. The 1181 bp product was digested by *Eco*RI and *Bam*HI, and cloned into pDEA-7Z (Shah *et al*., 1994) to produce pJYM4.2. Linear duplex DNA with 1.2 kb 3′ssDNA tails was produced by annealing *Eco*RI- and *Bam*HI-linearized pDEA-7Z to pJYM4.2 ssDNA. Gapped circular DNA products were purified by gel electrophoresis, electroeluted and repurified using Qiaquick DNA purification columns (Qiagen). The DNA was then linearized with *Xba*I, leaving an 18mer oligonucleotide on the complementary strand of the 1.2 kb 3′tail. This was then removed by heating at 50°C for 10 min and the tailed DNA was repurified. pPB4.3 gapped circular DNA (containing a 1.3 kb region of ssDNA) was prepared in a similar way by annealing *Eco*RI- and *Bam*HI-linearized pDEA-7Z to pPB4.3 ssDNA.

All DNA concentrations are expressed in moles of nucleotides.

Purification of hDmc1 protein

The *hDMC1* gene was PCR amplified from a human testis cDNA library and cloned into pET16b (Novagen) to generate phDMC1 in which the Dmc1 protein sequence was linked to a histidine tag. Recombinant hDmc1 protein was purified from 12 l of *E.coli* FB810 *recA*$^{-}$ pLysS (Benson *et al*., 1994) carrying plasmid phDMC1 grown at 30°C in tryptone phosphate media supplemented with 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol. At OD_{650} = 0.5, hDmc1 synthesis was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and, after 4 h, the cells (~70 g wet weight) were harvested by centrifugation, frozen in dry ice–ethanol and stored at –80°C. The cell paste was resuspended in 360 ml of extraction buffer [0.1 M Tris–HCl pH 8.0, 2 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol (DTT)] containing 0.2 M NaCl and the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mg/ml) and aprotinin (0.016 trypsin inhibitor units/ml). The suspension was divided into 30 ml aliquots, which were sonicated twice for 30 s, and Triton X-100 was added to 0.1%. Insoluble material was removed by centrifugation at 35 000 r.p.m. for 1 h in a Beckman 45 Ti rotor.

Proteins were precipitated from the clarified supernatant using 45% ammonium sulfate, and the pellet was resuspended in extraction buffer and dialyzed against 3 × 4.5 l of spermidine buffer (20 mM Tris–acetate pH 7.5, 7 mM spermidine–NaOH and 0.1 mM DTT) for 8 h. The precipitate containing hDmc1 was recovered by centrifugation and resuspended in 100 ml of T buffer (20 mM Tris–HCl pH 8.0, 0.5 M NaCl, 10% glycerol, 0.02% Triton X-100) containing 5 mM imidazole. Insoluble material was removed by centrifugation and the supernatant was loaded onto a 40 ml Talon column (Clontech). The column was washed successively with 400 and 150 ml of T buffer containing 30 and 50 mM imidazole, respectively, before hDmc1 was eluted with a 400 ml linear gradient of 0.05–1.0 M imidazole in T buffer. Fractions of hDmc1, which eluted as a broad peak, were identified by SDS–PAGE, pooled and dialyzed against R buffer (20 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM EDTA, 0.5 mM DTT) containing 125 mM KCl (R125) and loaded onto a 40 ml reactive blue column. The column was washed with 200 ml of R125 before a linear gradient of 0.125–1.5 M KCl in R buffer was applied. Fractions containing hDmc1 (peaking at 0.75 M KCl) were pooled, dialyzed against R125 and loaded onto a 40 ml heparin (Bio-Rad) column. The column was washed with 200 ml of R buffer containing 250 mM KCl and hDmc1 was eluted with a linear gradient of 0.25–1.0 M KCl in R buffer. hDmc1, eluting at ~0.4 M KCl, was dialyzed against R125 and loaded onto a 1 ml MonoQ column (HR 5/5) using a Pharmacia FPLC system. The column was washed with R125 before the hDmc1 was eluted using a linear gradient of 0.125–0.6 M KCl in R buffer. hDmc1 eluted in a sharp peak around 0.4 M KCl. The protein was dialyzed for 90 min against 2 l of S buffer (20 mM Tris–acetate pH 7.5, 0.2 M potassium acetate, 10% glycerol, 1 mM EDTA and 0.5 mM DTT) in Slide-A-Lyzer dialysis cassettes (Pierce) and stored in aliquots at –80°C. The final yield of hDmc1 was ~2 mg as determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard.

Recombinant hDmc1 protein was also produced from baculovirus-infected Sf9 cells, using the BAC-TO-BAC expression system (Gibco-BRL), and purified essentially as described above.

Purification of RecA, SSB, hRad51, hRad52 and hRPA

Escherichia coli RecA (Eggleston *et al*., 1997), hRad51 (Baumann *et al*., 1997), hRad52 (Benson *et al*., 1998) and hRP-A (Baumann and West, 1997) were purified as described. *Escherichia coli* SSB protein was purchased from Pharmacia.

Gel filtration

The molecular mass of purified hDmc1 protein (12 μg) was determined by comparison with gel filtration standards (bovine thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B_{12}, 1.35 kDa). Proteins were analyzed on a SMART system fitted with a 2.4 ml Superdex 200 PC 3.2/30 column (Pharmacia) equilibrated with S buffer. Forty 50 μl fractions were collected and analyzed by SDS–PAGE followed by Western blotting with a monoclonal anti-histidine antibody diluted at 1/5000 (Clontech).

DNA-binding assay

Reactions (20 μl) contained ^{32}P-labeled single-stranded ϕX174 DNA (1 μM) or nicked circular pFB585 duplex DNA (2 μM) in standard binding buffer [20 mM triethanolamine–HCl pH 7.5, 1 mM DTT, 2 mM ATP, 1 mM $Mg(OAc)_2$, 5% glycerol and 100 μg/ml BSA]. After 5 min at 37°C, hDmc1 or hRad51 were added and incubation was continued for a further 10 min. Complexes were fixed by addition of 0.2% glutaraldehyde followed by 15 min incubation at 37°C. Protein–DNA complexes were resolved by electrophoresis through 0.8% agarose gels in TAE buffer, dried onto filter paper and visualized by autoradiography.

Strand-transfer assay

Reactions (10 μl) contained single-stranded pPB4.3 DNA (10 μM) with hRad51 (3 μM) or hDmc1 (5 μM) in standard buffer [50 mM triethanolamine–HCl pH 7.5, 1 mM $Mg(OAc)_2$, 2 mM ATP, 1 mM DTT and 100 μg/ml BSA]. RecA reactions (3 μM) were carried out in 50 mM triethanolamine–HCl pH 7.5, 15 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 100 μg/ml BSA, 20 mM creatine phosphate and 2 U/ml creatine phosphokinase. In reactions containing RecA, SSB (0.5 μM) was added after RecA. For hRad51 or hDmc1 reactions, hRPA (0.18 μM) was added to the ssDNA first. After 5 min at 37°C, a mixture of ^{32}P-labeled nicked linear pPB4.3 (10 μM) and pPB284 (90 μM) heterologous duplex DNA was added and incubation was continued for 90 min. Reaction products were deproteinized by the addition of one-fifth volume of stop buffer (0.1 M Tris–HCl pH 7.5, 0.1 M $MgCl_2$, 3% SDS, 5 μg/ml ethidium bromide and 10 mg/ml proteinase K) followed by 20 min incubation at 37°C. Labeled DNA products were analyzed by electrophoresis through 0.8% TAE agarose gels run at 4.3 V/cm, dried onto filter paper and visualized by autoradiography. Where indicated, ATP was replaced by the same concentration of ATPγS, ADP or dATP.

Electron microscopy

Reactions (20 μl) contained pJYM4.2 ssDNA, nicked circular ϕX174 duplex DNA, pJYM4.2 tailed DNA or pPB4.3 gapped circular DNA in 20 mM triethanolamine–HCl pH 7.5, 1 mM DTT, 2 mM ATP, 1 mM $Mg(OAc)_2$. After 5 min at 37°C, hDmc1 was added and incubation was continued for a further 10 min. Protein–DNA complexes were fixed by addition of glutaraldehyde to 0.2% followed by 15 min incubation at 37°C. Samples were diluted and washed in 5 mM $Mg(OAc)_2$ prior to uranyl acetate staining (Sogo *et al*., 1987). Complexes were visualized at a magnification of 20 500× using a Philips C100 electron microscope. Complexes of RecA and nicked circular duplex DNA were prepared in the presence of ATPγS.

Yeast two-hybrid analysis

The full-length *hDMC1* and *hRAD51* genes were cloned into pGBDU-C3 and pGAD-C3, to produce fusions to the Gal4 DNA-binding and

activation domains. All fusions were confirmed by sequencing using a ABI377 automatic sequencer and by Western blotting using antibodies raised against the Gal4 DNA-binding and activation domains (Clontech). pJ69-4A was transformed and cells were plated on synthetic media lacking uracil, leucine and adenine or on media lacking uracil, leucine and histidine supplemented with 15, 30 and 45 mM 3-aminotriazole (James *et al*., 1996). Transformation, colony lift assays and liquid β-galactosidase assays were carried out according to the matchmaker kit manual (Clontech Laboratories).

Immunoprecipitation

Purified protein(s) (1 μg each) was incubated in S buffer for 30 min at 37°C. Complexes were then immunoprecipitated for 3 h at 4°C using 5 μg of monoclonal anti-hDmc1 mAbs (1B2, 1D12 or 4G3) covalently coupled to protein G–Sepharose beads. In some reactions, the anti-Dmc1 mAbs were replaced with anti-Rad52 polyclonal antibodies (pAbs). The beads were then washed three times with S buffer, boiled in SDS–loading buffer and proteins separated by SDS–PAGE. Western blotting was carried out using an anti-Rad51 mAb (3C10) that also recognizes hDmc1, or an anti-hDmc1 mAb (1D12).

For extract studies, the *hDMC1* and *hRAD51* genes were subcloned into pFASTBAC1 (Gibco-BRL) and recombinant baculoviruses were produced. Sf9 cells were infected with the *hDMC1* and *hRAD51* baculoviruses (m.o.i. 10) for 3 days at 27°C. Cells were harvested and resuspended in hypotonic buffer (10 mM Tris–HCl pH 7.5 , 1 mM EDTA) containing protease inhibitors and lysed using a Dounce homogenizer. Insoluble material was removed by high-speed centrifugation. The supernatant from $\sim 2 \times 10^7$ cells was pre-cleared using protein G–Sepharose for 1 h at 4°C and subjected to immunoprecipitation using hDmc1 mAb 1D12 in the presence of protein G–Sepharose beads (2 h at 4°C). Complexes were washed three times with 1% NP-40 containing 0.2 M NaCl or in hypotonic buffer containing NaCl, boiled in SDS-loading buffer and the proteins separated by SDS–PAGE. hRad51 was detected using the anti-hRad51 mAb 14B4 (GeneTex), which fails to cross-react with hDmc1 under these conditions. Proteins were detected by enhanced chemiluminescence (Amersham).

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