The Human Rad54 Recombinational DNA Repair Protein Is a Double-stranded DNA-dependent ATPase*

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Sigrid M. A. Swagemakers‡, Jeroen Essers‡, Jan de Wit‡, Jan H. J. Hoeijmakers‡,
and Roland Kanaar‡¶§

From the ‡Department of Cell Biology and Genetics, Erasmus University Rotterdam, P. O. Box 1738,
3000 DR Rotterdam, The Netherlands and the §Department of Radiotherapy, Division of Clinical Radiobiology, Dr. Daniël den Hoed Cancer Center, P. O. Box 5201, 3008 AE Rotterdam, The Netherlands

DNA double-strand break repair through the RAD52 homologous recombination pathway in the yeast Saccharomyces cerevisiae requires, among others, the RAD51, RAD82, and RAD54 genes. The biological importance of homologous recombination is underscored by the conservation of the RAD52 pathway from fungi to humans. The critical roles of the RAD52 group proteins in the early steps of recombination, the search for DNA homology and strand exchange, are now becoming apparent. Here, we report the purification of the human Rad54 protein. We showed that human Rad54 has ATPase activity that is absolutely dependent on double-stranded DNA. Unexpectedly, the ATPase activity appeared not absolutely required for the DNA repair function of human Rad54 in vivo. Despite the presence of amino acid sequence motifs that are conserved in a large family of DNA helicases, no helicase activity of human Rad54 was observed on a variety of different DNA substrates. Possible functions of human Rad54 in homologous recombination that couple the energy gained from ATP hydrolysis to translocation along DNA, rather than disruption of base pairing, are discussed.

DNA double-strand breaks (DSBs),1 generated by ionizing radiation and endogenously produced radicals, are extremely genotoxic lesions because as few as one or two unrepaired DSBs can lead to cell death (1). Therefore, it is not surprising that multiple pathways have evolved for the repair of DSBs (2, 3). Of the two main pathways, DNA end-joining uses no or extremely limited sequence homology to rejoin ends directly in a manner that need not be error-free, whereas homologous recombination requires extensive regions of DNA homology to repair DSBs accurately using information on the undamaged sister chromatid or homologous chromosome. The biological importance of DSB repair through homologous recombination is underscored by the conservation of its salient features from fungi to humans (4, 5).

Genetic experiments have established a role for at least nine genes of the yeast Saccharomyces cerevisiae in homologous recombination (6). These so-called RAD52 epistasis group genes include RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, and XRS2 (7–9). Mutations in any of these genes result in ionizing radiation-sensitive phenotypes. To date, proteins with amino acid sequence similarity to Rad50, Rad51, Rad52, Rad54, and Mre11 have been identified in mammals (3, 5). It is clear that the RAD52 homologous recombination pathway is functionally conserved from fungi to mammals. Biochemical experiments have demonstrated that the yeast and human Rad51 and Rad52 proteins perform key steps in homologous recombination, the search for DNA homology, and strand exchange, through similar mechanisms (9–20). Genetic experiments have shown that human RAD54 is the functional homolog of S. cerevisiae RAD54, because the human gene complements certain DNA repair phenotypes of S. cerevisiae rad54 cells (21). In addition, disruption of RAD54 in mouse embryonic stem (ES) cells and chicken DT40 cells impairs homologous recombination and results in ionizing radiation sensitivity (22, 23).

In addition to the biochemical activities of Rad51 and Rad52, the activities of other RAD52 group proteins, including Rad54, Rad55, and Rad57, are becoming apparent (9–20, 24–27). Here, we report the purification of the human Rad54 protein (hRad54) from baculovirus-infected insect cells. Rad54 contains seven amino acid sequence motifs that are conserved in a large superfamily of proteins (28), including DNA helicases involved in replication, recombination, and repair, such as the Escherichia coli DnaB, RuvB, and UvrD proteins. In particular, Rad54 belongs to the SWI2/SNF2 subfamily of ATPases (29). We show that hRad54 has ATPase activity that is absolutely dependent on double-stranded (ds) DNA. Unexpectedly, the ATPase activity of hRad54 is not absolutely required for its DNA repair function in vivo.

EXPERIMENTAL PROCEDURES

DNA Constructs—A cDNA construct encoding hRad54 containing a polyhistidine amino-terminal tag (MGSSHHHHHSGGGLVPGRSH) and a carboxyl-terminal hemagglutinin tag (VTYPYDVPDYAS) was generated. The sequence of all DNA fragments produced by polymerase chain reaction was confirmed by sequence analysis. For expression in mouse ES cells, the cDNA was placed under control of the phosphoglycerate kinase promoter. A construct expressing a tagged version of Rad54 was amplified from mouse ES cells, the cDNA was placed under control of the phosphoglycerate kinase promoter. A construct expressing a tagged version of Rad54 was amplified from mouse ES cells, the cDNA was placed under control of the phosphoglycerate kinase promoter.

ES Cell Culture—E14 ES cells were cultured and electroporated with the following DNA constructs. DNA in the form of supercoiled plasmids (10 μg/106 cells) was transfected into E14 ES cells by electroporation in the presence of a 10 μg/ml concentration of polybrene (Sigma Chemical Co.).

1 The abbreviations used are: DSB, double-strand break; ES, embryonic stem; hRad54, human Rad54; ds, double-stranded; ss, single-stranded; bp, base pair(s).

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‡ Fellow of the Royal Netherlands Academy of Arts and Sciences. To whom correspondence should be addressed: Dept. of Cell Biology and Genetics, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 31-10-408-7168; Fax: 31-10-436-0225; E-mail kanar@pop.fgg.eur.nl.

¶ Department of Radiotherapy, Division of Clinical Radiobiology, Dr. Daniël den Hoed Cancer Center, P. O. Box 5201, 3008 AE Rotterdam, The Netherlands.
mRAD54

The disrupted mRAD54 allelic in this line contain the neomycin- and puromycin-selectable markers, respectively. The constructs were co-electroporated with a plasmid carrying the hygromycin-selectable marker. Clones were selected as described and screened for hRAD54 expression by immunoblot analysis.

The colony-forming ability of the ES cell lines after irradiation with a 137Cs source as described. Cloning efficiencies varied from 10 to 30%. Cells were grown for 7 days, fixed, stained, and counted. All measurements were performed in triplicate.

Purification of hRad54 Protein—The cDNAs encoding the tagged hRad54 and hRad54 constructs were subcloned into pFastBac1 (BAC-TO-BAC Baculovirus Expression System, Life Technologies, Inc.). The resulting plasmids were transformed into DH10Bac E. coli cells to allow site-specific transposition into bacmid pMON14272. High molecular weight recombinant bacmids were isolated and transfected into SF21 cells to produce virus stocks that were amplified as described by the manufacturer. For protein production, 4.5 × 10^7 SF21 cells were infected with the recombinant baculoviruses at a multiplicity of infection of 15. The ES cell lines were selected for resistance to both neomycin and puromycin. The putative Walker A nucleotide binding motif and was changed to an arginine residue using site-directed mutagenesis. The resulting hRad54 cDNA was expressed as a protein, we generated a cDNA expression construct encoding a hRad54 protein, we generated a cDNA expression construct encoding a hRad54 protein, we generated a cDNA expression construct encoding a hRad54 protein.

RESULTS AND DISCUSSION

Purification of the Human Rad54 Protein—For the purpose of purification, identification, and protein-protein interactions studies, we constructed a cDNA expressing hRad54 containing an amino-terminal polyhistidine tag and a carboxyl-terminal hemagglutin tag. The addition of the tags did not interfere with the biological function of hRad54. However, the presentation and discussion of those results is deferred until Fig. 3. In addition to the cDNA expressing wild-type tagged hRad54 protein, we generated a cDNA expression construct encoding a tagged version of hRad54 containing a single amino acid substitution at position 189. This invariant lysine residue is in the putative Walker A nucleotide binding motif and was changed to an arginine residue using site-directed mutagenesis. The resulting protein is referred to as hRad54K189R. For a number of ATPases, including E. coli Uvrd and S. cerevisiae Rad3, version of the equivalent lysine residue into an arginine residue severely impairs nucleotide triphosphate hydrolysis (34, 35). However, for Uvrd and Rad3, nucleotide binding is unaffected by the mutation, implying that the overall structure of the protein remains intact.

For protein production, we placed both cDNAs under transcriptional control of the polyhedrin promoter in recombinant baculoviruses. These viruses were used to infect SF21 cells. A Coomassie-stained SDS-polyacrylamide gel containing crude extract of the cells infected with the hRad54 expressing baculovirus is shown in Fig. 1, lane 2. Immunoblot analysis demonstrated that the prominent band between the 107- and 68-kDa molecular mass markers is the hRad54 protein (data not shown). The extract was subsequently fractionated over phosphocellulose, Ni2+-nitrilotriacetate agarose, and Mono S column. The sequence of the three oligonucleotides used was as follows: oligonucleotide a, 5′-CCAAA-GCTTGCAATGGCAAGGTGTAGCTACCTAGAGA; oligonucleotide b, 5′-TTTGTGCAGCGTACCCAACTGTCCTAGCCTCGCTACCTAGAGA; oligonucleotide c, 5′-CCAGCTTGATCGTCGTCAGGTCGACCTCTAGAGAGGGCCATAGGCTCC. Annecing of oligonucleotide a resulted in a 35-bp duplex, whereas annealing of oligonucleotides b and c resulted in 15-bp and 35-bp duplexes, respectively.

The gel-purified oligonucleotides were 5′-end labeled using T4 polynucleotide kinase and [γ-32P]ATP and annealed to M31mp18 viral DNA. Labeled substrates were separated from labeled oligonucleotides by gel filtration through a Sepharose CL-4B column.

Branch cut-ended 78-bp dsDNA substrate was made by isolating the XmnI-BglII restriction fragment from the hRad54 cDNA (21) and treating it with Klenow DNA polymerase in the presence of dTTP, dGTP, dCTP, and [α-32P]dATP. This procedure resulted in incorporation of radiolabel in one of the two strands, indicated by the underlined nucleotide in italics; 5′-CCGCGTCTGGGAGATGTTCAAAAGAGAAGAGCTCTGCTATATCTCTACCGCTGTGAGGGACCATTGAGGA-GAACAGT.

Branch cut-ended oligonucleotide substrates were made by annealing three partially complementary oligonucleotides. The sequences of the oligonucleotides (designated oligonucleotides 1–3) were the same as those used to investigate the DNA helicase activities of the E. coli RecG and T4 Uvrd proteins (31, 32). The arms of the branched structure were between 24 and 26 nucleotides, and the annealing and purification procedures were as described (33). In the experiment shown, oligonucleotide 2 was 5′-end labeled with the use of T4 polynucleotide kinase and [γ-32P]ATP.

All DNA substrates were incubated for 60 min at 30 °C. Protein concentrations were varied from 0 to 0.1 μM, and ATP concentrations were varied from 0.2 to 2 μM. Reactions that were carried out in the presence of an ATP regeneration system contained 40 mM phosphocreatine and 10 units/ml creatine phosphokinase. Reactions were terminated by the addition of SDS and EDTA to 0.2% and 20 mM, respectively. DNA species were separated by electrophoresis through non-denaturing polyacrylamide gels that were dried and analyzed by autoradiography.

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hRad54 preparation had a purity of approximately 90%. A sample of the final purification step of the hRad54 K189R protein, which was produced and purified in exactly the same manner as the wild-type protein, is shown in Fig. 1, lane 6.

The Human Rad54 Protein Is a dsDNA-dependent ATPase—We tested whether the purified hRad54 protein could hydrolyze ATP, because it contains Walker A and B amino acid sequence motifs that are involved in ATP hydrolysis in a large number of proteins (36). Increasing amounts of hRad54 protein were incubated with ATP for 60 min at 30 °C. Released radiolabeled phosphate was separated from nonhydrolyzed ATP by thin layer chromatography, and the extent of hydrolysis was quantitated (Fig. 2). In the absence of DNA, no significant hydrolysis of input ATP was observed. The amount of input ATP hydrolyzed varied between 0.2 and 1.0% and did not increase with increasing protein concentration. Because the ATPase activity of the SWI2/SNF2 protein is stimulated by DNA (37), we included dsDNA in the reaction mixture. Fig. 2A shows that inclusion of dsDNA is absolutely required for the activation of the ATPase activity of hRad54. Recently, it was shown that, like hRad54, the S. cerevisiae Rad54 protein also possesses DNA-dependent ATPase activity (26). The observed ATPase activity of hRad54 was not due to a contaminating ATPase, because the identical preparation of hRad54 K189R protein displayed no ATPase activity in the presence of dsDNA. The lack of ATPase activity of hRad54 K189R is not due to a defect in DNA binding, because analysis of the DNA binding properties showed that the wild-type and mutant proteins had similar affinities for both ds and single-stranded (ss) DNA (data not shown).

We next analyzed the effect of ssDNA on hRad54-mediated ATP hydrolysis. M13 viral DNA also stimulated the hRad54 ATPase activity but to a lesser extent than dsDNA (Fig. 2A). Because M13 viral DNA has a significant amount of secondary structure and given the fact that dsDNA is such an effective stimulator of the hRad54 ATPase activity, we tested whether...
TABLE I

<table>
<thead>
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</tr>
<tr>
<td>Poly(dA)</td>
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<tr>
<td>RNA</td>
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ssDNA that does not form secondary structure can stimulate the ATPase activity. Therefore, we incubated increasing amounts of hRad54 with poly(dA) or poly(dT). Both homopolymers were unable to activate the ATPase activity of hRad54 (Fig. 2B). The measured ATPase activity in the presence of poly(dA) and poly(dT) was 0.1–0.5% and 0.3–0.9%, respectively. However, when the two polymers were allowed to form base pairs before addition to the reaction mixture, they did efficiently activate the hRad54 ATPase activity (Fig. 2B). We conclude that hRad54 is a dsDNA-dependent ATPase and that the substitution of amino acid 189 from lysine to arginine results in loss of the ATPase activity.

The rate of ATP hydrolysis by hRad54 was determined in the presence of dsDNA (Fig. 2C). The turnover rate was found to be ~800 mol of ATP min$^{-1}$ mol$^{-1}$ of hRad54. Thus, hRad54 is a more active ATPase than other DNA-dependent ATPases involved in recombination, such as human Rad51, a DNA strand exchange protein that exhibits ssDNA-stimulated ATPase activity with a turnover rate of 0.16 min$^{-1}$ (14). The hRad54 ATPase exhibits a turnover rate within the range of DNA helicases involved in recombination and repair, such as RuvB (4.2 min$^{-1}$) and UvrD (10,000 min$^{-1}$) (35, 38).

We varied a number of reaction conditions to determine some requirements for hRad54-mediated ATP hydrolysis. The results of these experiments are summarized in Table I. In addition to dsDNA, divalent cations were found to be essential for ATP hydrolysis. The topology of the dsDNA cofactor is not critical, because duplex linear and supercoiled DNA stimulated the ATPase activity of hRad54 to similar levels. Like ssDNA, RNA did not activate the hRad54 ATPase activity. Optimal catalytic activity was obtained at pH 7.0 and at a temperature of 25–30 °C.

Amino- and Carboxyl-terminal Tags Do Not Interfere with the Biological Function of hRad54—Before analyzing other activities of the hRad54 protein, we determined whether the tags on the protein would interfere with its function. mRAD54 knockout cells were electroporated with the cDNA constructs expressing tagged hRad54 and hRad54$^{K189R}$. For each construct, 24 clones were analyzed for protein expression by immunoblot analysis using anti-hRad54 antibodies. A number of independent cell lines were identified that expressed either tagged wild-type or mutant protein. Expression levels, which did not vary much between the different clones, ranged from one-half to twice the level found in wild-type ES cells. An example of an immunoblot containing protein extracts from mRAD54 knockout ES cell lines expressing tagged versions of the wild-type and mutant hRad54 proteins is shown in Fig. 3A.

We have shown previously that mRAD54 knockout ES cells are sensitive to ionizing radiation and the DNA cross-linking agent mitomycin C. In addition, the mRad54 protein could rescue the mitomycin C sensitivity (22). Therefore, we tested whether the mRAD54 knockout cell lines that expressed the tagged wild-type and mutant hRad54 proteins could rescue the γ-ray and mitomycin C sensitivity. Fig. 3B shows that the wild-type tagged hRad54 completely corrects the γ-ray sensitivity caused by the mutations in mRAD54. The efficiency of the rescue did not depend on the expression level of hRad54 because a cell line expressing one-half the level of Rad54 found in wild-type cells gave similar results (data not shown).

Interestingly, expression of the tagged hRad54$^{K189R}$ protein resulted in a partial rescue of the γ-ray sensitivity (Fig. 3B). As above, the expression level of hRad54$^{K189R}$ was critical to the observed effect because an independently obtained cell line, expressing twice the level of Rad54 compared with wild-type cells, gave similar results (data not shown). Tagged hRad54 also rescued the mitomycin C sensitivity of mRAD54 knockout cells (Fig. 3C). Again, expression of tagged hRad54$^{K189R}$ led to a partial rescue. Similar results were obtained with independent clones expressing different levels of tagged hRad54 and hRad54$^{K189R}$ respectively (data not shown). We conclude that the amino-terminal polyhistidine tag and the carboxyl-terminal hemagglutinin tag do not interfere with the function of the hRad54 protein.

Rad54, together with SWI2/SNF2 and Mot1, belongs to the SNF2/SWI2 family of DNA-stimulated ATPases (29). The ATPase activity of the SNF2/SWI2 and Mot1 proteins is essential for their functions in vivo (37, 39). Surprisingly, we found that the hRad54$^{K189R}$ protein, which is completely deficient in ATPase activity (Fig. 2A), is partially functional in vivo (Fig. 3, B and C). Thus, ATP hydrolysis can, at least in part, be uncoupled from other biological activities of hRad54, implying that other properties of the protein are also important for its function. One of those functions could be in contributing to the formation of multiprotein complexes. The absence of one of the components of such complexes might be more detrimental than the presence of a crippled component. For example, the absence of one of the components of the ERCC1/XPF structure-specific endonuclease causes instability of the other component (40, 41). Because genetic and physical interactions have been detected among many of the RAD52 group genes and proteins (25, 26, 42–48), it is likely that these proteins function in the context of complex protein machines. Similar to the observation presented here for hRad54, an ATPase-deficient mutant of the transcription-coupled nucleotide excision repair protein CSB, which is part of a multiprotein complex, is partially active in vivo as well (49).

Analysis of hRad54 Activity in DNA Helicase Assays—Because the hRad54 protein contains seven amino acid sequence motifs that are found in many DNA helicases and because it has DNA-dependent ATPase activity, we tested whether hRad54 could use the energy gained from ATP hydrolysis to disrupt base pairing in duplex DNA. We tested five distinct DNA substrates in helicase assays, because DNA helicases differ in their substrate specificity. The first set of three substrates consisted of M13 viral DNA to which oligonucleotides were annealed. One substrate contained a 35-bp duplex region,
FIG. 3. hRad54 rescues the γ-ray and mitomycin C sensitivity of mRAD54 knockout ES cells. A, immunoblot of protein extracts from wild-type (+/+ ) and mRAD54 knockout (−/− ) ES cells. mRAD54 knockout ES cells were stably transfected with the wild-type (hRAD54) or mutant (hRAD54K189R) cDNA constructs expressing the tagged proteins. Protein extracts from the indicated cell lines were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with affinity-purified anti-hRad54 antibodies. Detection was with alkaline phosphatase-coupled goat anti-rabbit antibodies. The positions of the 107- and 68-kDa protein molecular mass markers are indicated on the left. The position of the mammalian Rad54 protein is indicated on the right. B, clonogenic survival assay of the wild-type, mRAD54 knockout, and the radiolabeled reaction product are indicated to the left. The positions of the substrate and the radiolabeled reaction product are indicated to the left. The asterisk denotes the position of the 32P label. The three control reactions were included. The positions of the substrate and the radiolabeled reaction product are indicated to the left.

FIG. 4. The hRad54 protein displays no activity in DNA helicase assays. A, DNA helicase assay using a 35-nucleotide (nt) oligomer annealed to M13 viral DNA as a substrate. The three oligonucleotides used were either completely complementary or contained a 15-nucleotide 5’ or 3’ noncomplementary region, in addition to the 35 complementary nucleotides. Oligonucleotides were labeled with 32P at their 5’-end before annealing to the viral DNA. DNA substrates (−20 pmol) were incubated with 100 ng of hRad54 or hRad54K189R protein in the presence of ATP for 60 min at 30 °C. Products were separated by electrophoresis through a nondenaturing polyacrylamide gel and visualized by autoradiography. The positions of the substrate and the radiolabeled reaction products are indicated to the right of the autoradiogram. The asterisk denotes the position of the 32P label. The three control reactions shown either lacked hRad54 protein (untreated), were heated to 95 °C (ΔT), or contained UvrD protein (UvrD). B, DNA helicase assay using a 79-bp linear dsDNA as substrate. The DNA substrate (−20 pmol) was incubated with 100 ng of hRad54 or hRad54K189R protein for 60 min at 30 °C. Reactions were carried out in the absence or presence of ATP or in the presence of an ATP regeneration system (ATP reg.), as indicated. They were analyzed as described in A, and the same control reactions were included. The positions of the substrate and the radiolabeled reaction product are indicated to the left.

whereas the others were forked substrates that contained 15-nucleotide noncomplementary regions either 5’ or 3’ of the 35-bp duplex region. The hRad54 protein appeared unable to displace the oligonucleotide from the M13 DNA (Fig. 4A). The hRad54 protein concentration was varied over the same range as was used for the ATPase assays shown in Fig. 2 (data not shown). The lack of helicase activity cannot be explained by a rapid depletion of ATP, because the inclusion of an ATP regeneration system in the reaction mixture did not result in detectable helicase activity (data not shown). In contrast, the UvrD protein efficiently displaced the oligonucleotide from all three substrates (Fig. 4A). Our results with hRad54 are consistent with those obtained with the S. cerevisiae Rad54 protein, which also displays no helicase activity on substrates containing noncomplementary ssDNA tails (26).

In E. coli, the RecBCD DNA helicase plays a pivotal role in processing DSBs during recombination (50). Its substrate is a dsDNA end. Given the involvement of Rad54 in recombinational DSB repair, it was important to test whether the protein exhibited DNA helicase activity on a blunt-ended dsDNA substrate (Fig. 4B). We were unable to detect any helicase activity of hRad54 on this substrate, even in the presence of an ATP regeneration system and over a wide range of hRad54 concentration. In contrast, UvrD efficiently separated the strands this
DNA substrate (Fig. 4B). Some DNA helicases, such as the E. coli RecA protein, which is involved in recombination and repair, are inactive on forked duplexes and blunt-ended duplex DNA substrates, such as those tested in Fig. 4, A and B. Instead, the RecG protein unwinds substrates containing a three-way duplex branch (31). However, even with this DNA substrate, no helicase activity of hRad54 was detected (Fig. 4C).

On the basis of its amino acid sequence, Rad54 belongs to a superfamily of DNA-dependent ATPases (28). Many superfamily members have DNA helicase activity. Although the experiments presented above do not rule out the possibility that hRad54 has DNA helicase activity, they make it less likely. Possibly, the seven conserved amino acid sequence motifs that define the superfamily provide a general activity, of which helicase activity could be a subset (51). This more general activity could be the ability to translocate along DNA at the expense of ATP hydrolysis (52). By using the energy gained from ATP hydrolysis translocation of Rad54 might be useful in at least three stages of homologous recombination. First, in light of the interaction between the Rad54 and Rad51 proteins (26, 45, 47, 48), translocation of Rad54 might provide processivity to Rad51-mediated DNA strand exchange. Compared with the E. coli RecA protein, hRad51 makes short heteroduplex joints (9). A role for Rad54 in extending these joint regions is consistent with the recent demonstration that S. cerevisiae Rad54 stabilizes D-loops (26). Providing processivity to joint formation might be especially important in the context of chromatin. Second, translocation of hRad54 could be to promote branch migration of Holliday junctions to extend heteroduplex DNA, in a manner that is analogous to the molecular motor function of the RuvB protein. Third, an alternative function of Rad54 could be in removing the Rad51 protein from joint molecules, formed by the two recombining partners after the initiation of recombination, in order to prevent reversal of the reaction. In this respect, the action of Rad54 would be analogous to that of the Mot1 protein, which disrupts protein DNA complexes involved in transcription (35). The availability of purified hRad54 protein facilitates testing of the possible functions of the protein described above.

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