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

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**DNA double-strand break repair through the *RAD52* homologous recombination pathway in the yeast *Saccharomyces cerevisiae* requires, among others, the *RAD51*, *RAD52*, 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*Δ 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 (MGSSHHHHHHSSGLVPRGSH) and a carboxyl-terminal hemagglutin 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 hRad54 containing a single amino acid substitution at position 189 was also generated. The invariant lysine residue at this position, which is in the putative GKT Walker-type nucleotide binding motif, was changed to an arginine residue (21). This protein is referred to as $hRad54^{K189R}$.

*ES Cell Culture*—E14 ES cells were cultured and electroporated with DNA constructs as described (30). The hRad54 expressing constructs described above were electroporated into *mRAD54* knockout ES line

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[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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*mRAD54*$^{307neo/pur}$ (22). The disrupted *mRAD54* alleles 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 (22) and screened for hRad54 expression by immunoblot analysis.

The sensitivity of ES cells to increasing doses of ionizing radiation and mitomycin C was determined by measuring their colony-forming ability. After trypsinization and counting, various dilutions of the different ES cell lines were placed into gelatinized 60-mm dishes, and after 12–24 h, cells were incubated for 1 h in mitomycin C-containing medium. Ionizing radiation sensitivity was determined by comparing the colony-forming ability of the ES cell lines after irradiation with a $^{137}$Cs source as described (22). 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$^{K189R}$ proteins 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 bMON14272. 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, $4.5 \times 10^8$ Sf21 cells were infected with the recombinant baculoviruses at a multiplicity of infection of 10. Two days postinfection, cells were collected by low speed centrifugation and washed twice with ice-cold phosphate-buffered saline. For fractionation, the cells were lysed in eight packed cell volumes of ice-cold Buffer A (20 mM Tris-HCl (pH 9.0), 300 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml antipain, 1 $\mu$g/ml pepstatin A, and 1 $\mu$g/ml chymostatin) for 30 min on ice. After clarification of the lysate by centrifugation, the supernatant was neutralized with four equivalents of packed cell volumes of ice-cold Buffer B (100 mM Tris-HCl (pH 6.8), 300 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml antipain, 1 $\mu$g/ml pepstatin A, and 1 $\mu$g/ml chymostatin). This crude extract (fraction I) was diluted with 0.5 volume of Buffer C (20 mM Hepes-KOH (pH 8.0), 0.2 mM EDTA, 2 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 mM $\beta$-mercaptoethanol), loaded onto a phosphocellulose column (Whatman P11), and equilibrated with Buffer C containing 0.2 M KCl, and after washing, bound proteins were eluted with Buffer C containing 1.0 M KCl (fraction II). Imidazole-HCl (pH 7.9) was added to a final concentration of 2 mM to fraction II, which was subsequently incubated overnight at 4 °C with 1 ml $Ni^{2+}$-nitrilotriacetate agarose (Qiagen). The resin was washed with Buffer D (20 mM Tris-HCl (pH 7.5), 1 mM $\beta$-mercaptoethanol, 0.05% Nonidet P-40, 10% glycerol, 500 mM KCl, and 20 mM imidazole-HCl (pH 7.9)). The hRad54 proteins were eluted with Buffer E (20 mM Tris-HCl (pH 7.5), 1 mM $\beta$-mercaptoethanol, 0.05% Nonidet P-40, 10% glycerol, 200 mM KCl, 1 mM EDTA, and 200 mM imidazole-HCl (pH 7.9)) to yield fraction III. Fraction III was diluted with three volumes of Buffer F (20 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.01% Nonidet P-40) and loaded onto a Mono S column (HR5/5) equilibrated with Buffer F containing 0.2 M KCl. The column was washed with 5 column volumes of equilibration buffer, and the hRad54 proteins were eluted with a 20-column volume linear gradient from 0.2 to 1.0 M KCl in Buffer F. The proteins (fraction IV) eluted around 0.4 M KCl. Aliquots were frozen in liquid $N_2$ and stored at −80 °C. The yield from $4.5 \times 10^8$ infected Sf21 cells varied between 60 and 120 $\mu$g of hRad54 protein.

*ATPase Assay*—Standard reaction mixtures contained 20 mM $KPO_4$ (pH 7.0), 4 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 100 $\mu$g/ml bovine serum albumin, 200 $\mu$M ATP, 0.25 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (>5000 Ci/mmol), 45 $\mu$M DNA (concentration in nucleotides) and 0–100 ng of hRad54 or hRad$^{K189R}$ protein in a 10-$\mu$l volume. Incubations were for 60 min at 30 °C. Reactions were initiated by the addition of DNA and $MgCl_2$ to a mixture containing the other components and terminated by the addition of EDTA to 167 mM. Released phosphate was separated from ATP by thin-layer chromatography on polyethyleneimine cellulose using 0.75 M $KH_2PO_4$ as running buffer. Hydrolysis was quantitated with the use of a Molecular Dynamics PhosphorImager. Background hydrolysis observed in the absence of protein (~2%) was subtracted. Fig. 2 displays the results of three or four independent experiments. To determine the optimal pH of the reaction, the following buffers were used: Bis-Tris (pH 6.0), $KPO_4$ (pH 7.0), and Tris (pH 7.5, 8.0, and 8.5) (see Table I).

*DNA Helicase Assay*—Partially dsDNA substrates were generated by annealing oligonucleotides to M13mp18 viral DNA. The sequence of the three oligonucleotides used was as follows: oligonucleotide a, 5′-CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGA; oligonucleotide b, 5′-TTTGCTGCCGGTCACCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGA; and oligonucleotide c, 5′-CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGAAGGGGCCCATGGCTC. Annealing of oligonucleotide a resulted in a 35-bp duplex, whereas annealing of oligonucleotides b and c resulted in 15-nucleotide overhangs, either 5′ or 3′, in addition to the 35-bp duplex region. The gel-purified oligonucleotides were 5′-end-labeled using T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP and annealed to M13mp18 viral DNA. Labeled substrates were separated from labeled oligonucleotides by gel filtration through a Sepharose CL-4B column.

A blunt-ended 79-bp dsDNA substrate was prepared by isolating the *Xma*I-*Bgl*II restriction fragment from the *hRAD54* cDNA (21) and treating it with Klenow DNA polymerase in the presence of dTTP, dGTP, dCTP, and [$\alpha$-$^{32}$P]dATP. This procedure resulted in incorporation of radiolabel in one of the two strands, indicated by the underlined nucleotide in italics; 5′-CCGGGTCTGGCGAGATGGTCAAAAGAAGACTTGCTATATCTACCGCCTGCTGTCTGCAGGGACCATTGAGGAGAAG*A*TC.

Branched dsDNA 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 UvsW proteins (31, 32). The arms of the branched structure were between 24 and 26 bp. The purification and annealing procedures were as described (33). In the experiment shown, oligonucleotide 2 was 5′-end-labeled with the use of T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP.

All DNA substrates were incubated for 60 min at 30 °C. Protein concentrations were varied from 0 to 0.1 $\mu$M, and ATP concentrations were varied from 0.2 to 2 mM. 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 nondenaturing polyacrylamide gels that were dried and analyzed by autoradiography.

## 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 hRad54$^{K189R}$. For a number of ATPases, including *E. coli* UvrD and *S. cerevisiae* Rad3, conversion 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 encoding 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, $Ni^{2+}$-nitrilotriacetate agarose, and Mono S columns as described under "Experimental Procedures." Samples of the hRad54-containing fractions were analyzed by electrophoresis through an SDS-polyacrylamide gel that was stained with Coomassie Blue (Fig. 1). We estimate that the final

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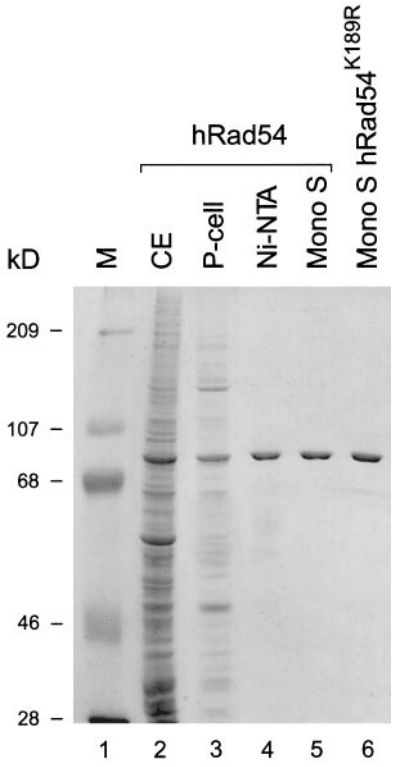


FIG. 1. **Purification of the hRad54 protein.** Coomassie-stained SDS-polyacrylamide gel containing samples taken at different stages of the hRad54 purification. A protein extract from Sf21 cells infected with a baculovirus expressing hRad54 (*CE*) (*lane 2*) was fractionated sequentially over phosphocellulose (*P-cell*) (*lane 3*), $ni^{2+}$-nitrilotriacetate agarose (*Ni-NTA*) (*lane 4*), and Mono S (*lane 5*) columns. The $hRad54^{K189R}$ protein was purified by the same method, and a sample of the final preparation is shown in *lane 6*. *Lanes 4–6* contain approximately 0.4 $\mu$g of protein. The size of the protein molecular mass markers (*M*) in *lane 1* is indicated in kDa.

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. 2*A* 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).

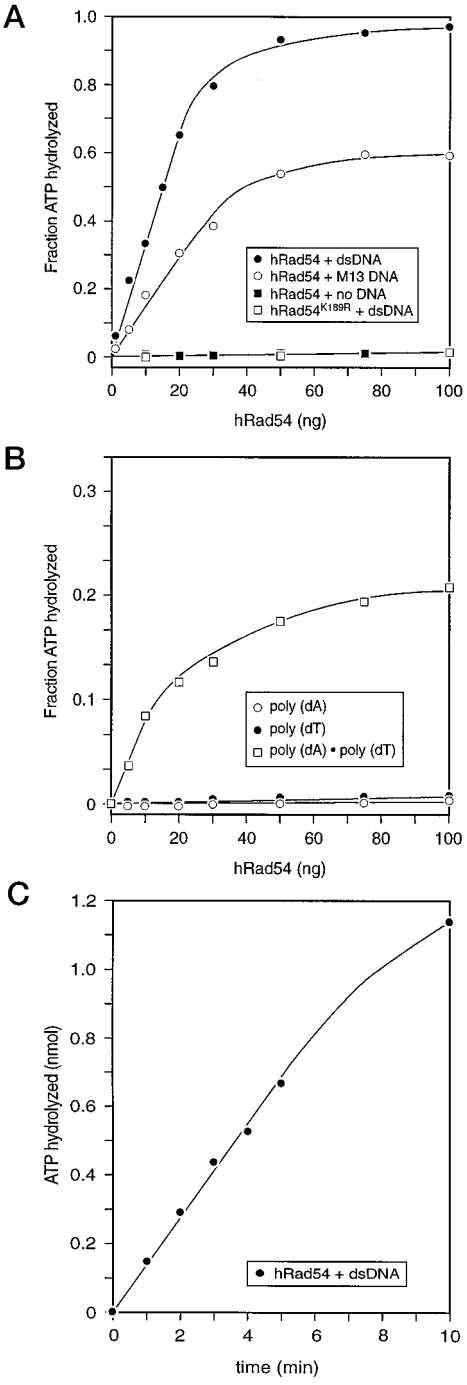


FIG. 2. **The hRad54 protein is a dsDNA-dependent ATPase.** *A*, the amount of ATP hydrolyzed by hRad54 and $hRad54^{K189R}$ after 60 min in the presence and absence of DNA is displayed as a function of enzyme concentration. The amount of ATP in the reaction mixture at time 0 was 2 nmol. ATPase assays were carried out as described under "Experimental Procedures." Reaction mixtures (10 $\mu$l) contained 0–100 ng of hRad54. *B*, as in *A* except that poly(dA), poly(dT), or hybridized poly(dA-dT) was used as DNA cofactor. *C*, kinetics of ATP hydrolysis by hRad54. Aliquots were removed from a reaction mixture at the indicated time points, and the extent of ATP hydrolysis by hRad54 in the presence of dsDNA was measured. The concentration of hRad54 in the reaction corresponded to the 15-ng data point in *A*.

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. 2*A*). 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

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TABLE I
*ATPase activity of the hRad54 protein*

The ATPase activity of 20 ng of hRad54 was determined under standard conditions described under "Experimental Procedures," with the exception of the indicated variable. An ATPase activity of 100% corresponds to hydrolysis of 65% of input ATP.

| Variable | Relative ATPase activity |
|---|---|
| | % |
| Divalent cation | |
| None | <1 |
| $Mg^{2+}$ | 100 |
| $Mn^{2+}$ | 109 |
| pH | |
| 6.0 | 21 |
| 7.0 | 100 |
| 7.5 | 83 |
| 8.0 | 44 |
| 8.5 | 14 |
| Temperature | |
| 25 °C | 124 |
| 30 °C | 100 |
| 37 °C | 34 |
| Nucleic acid cofactor | |
| None | <1 |
| Supercoiled DNA | 100 |
| Linear dsDNA | 90 |
| M13 viral DNA | 48 |
| Poly(dT) | <1 |
| Poly(dA) | <1 |
| RNA | <1 |

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. 2*B*). 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. 2*B*). 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. 2*C*). The turnover rate was found to be ~800 mol of ATP $min^{-1}$ $mol^{-1}$ 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 $hRad54^{K189R}$ proteins. 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. 3*A*.

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. 3*B* 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 hRad54K189R protein resulted in a partial rescue of the γ-ray sensitivity (Fig. 3*B*). As above, the expression level of $hRad54^{K189R}$ was not 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. 3*C*). 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 hemagglutin 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. 2*A*), 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,

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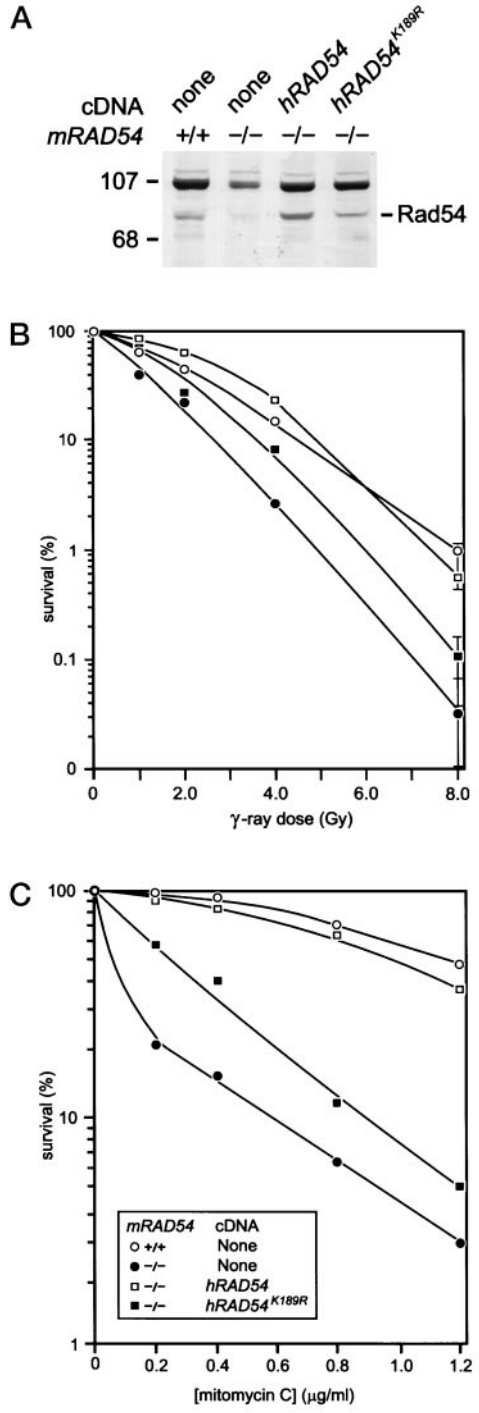


FIG. 3. **hRad54 rescues the γ-ray and mitomycin C sensitivity of *mRAD54* knockout ES cells.** *A,* immunoblot of protein extracts from wild-type (+/+) and *mRAD54* knockout (–/–) ES cells. *mRAD54* knockout ES cells were stably transfected with the wild-type (*hRAD54*) or mutant (*hRAD54*$^{K189R}$) 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 cDNA-transfected ES cell lines after treatment with increasing doses of γ-rays. The percentage of surviving cells as measured by their colony-forming ability is plotted as a function of the γ-ray dose. Details of the protocol are described under "Experimental Procedures." The S.E. values are not indicated, except for the dose of 8 Gy, because for all other doses they were within 4–20%. *C,* clonogenic survival assay of the same cell lines shown in *B* after treatment with increasing concentrations of mitomycin C.

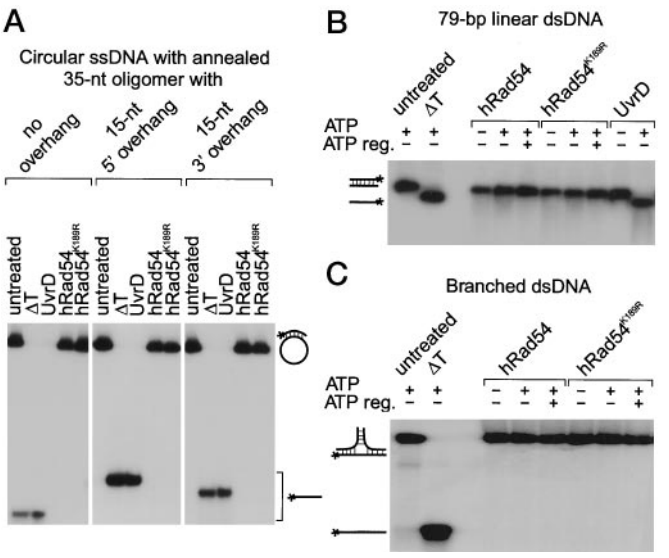


FIG. 4. **The hRad54 protein displays no activity in DNA helicase assays.** *A,* DNA helicase assay using a 35-nucleotide (*nt*) oligomer annealed to M13 viral DNA as a substrate. The three oligonucleotides used were either completely complementary or contained a 15-nucleotide 5′ or 3′ noncomplementary region, in addition to the 35 complementary nucleotides. Oligonucleotides were labeled with $^{32}$P at their 5′-end before annealing to the viral DNA. DNA substrates (~20 pmol) were incubated with 100 ng of hRad54 or hRad54$^{K189R}$ 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 $^{32}$P label. The three control reactions shown either lacked hRad54 protein (*untreated*), were heated to 95 °C (Δ*T*), 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 hRad54$^{K189R}$ 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* of the autoradiogram. *C,* DNA helicase assay using a branched dsDNA as substrate. The experiment was performed as described in *B*, except that the DNA substrate was a branched dsDNA containing arms of 24–26 bp that was generated by annealing three partially complementary oligonucleotides.

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. 4*A*). 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. 4*A*). 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. 4*B*). 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

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DNA substrate (Fig. 4*B*).

Some DNA helicases, such as the *E. coli* RecG 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. 4*C*).

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 along DNA 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 joints 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 between 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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