THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 23, Issue of June 4, pp. 24081–24088, 2004

Printed in U.S.A.

Effects of Tumor-associated Mutations on Rad54 Functions*

Received for publication, March 10, 2004, and in revised form, March 30, 2004
Published, JBC Papers in Press, March 31, 2004, DOI 10.1074/jbc.M402719200

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Yeast *RAD54* gene, a member of the *RAD52* epistasis group, plays an important role in homologous recombination and DNA double strand break repair. Rad54 belongs to the Snf2/Swi2 protein family, and it possesses a robust DNA-dependent ATPase activity, uses free energy from ATP hydrolysis to supercoil DNA, and cooperates with the Rad51 recombinase in DNA joint formation. There are two *RAD54*-homologous genes in human cells, *hRAD54* and *RAD54B*. Mutations in these human genes have been found in tumors. These tumor-associated mutations map to conserved regions of the hRad54 and hRad54B proteins. Here we introduced the equivalent mutations into the *Saccharomyces cerevisiae RAD54* gene in an effort to examine the functional consequences of these gene changes. One mutant, *rad54 G484R*, showed sensitivity to DNA-damaging agents and reduced homologous recombination rates, indicating a loss of function. Even though the purified rad54 G484R mutant protein retained the ability to bind DNA and interact with Rad51, it was nearly devoid of ATPase activity and was similarly defective in DNA supercoiling and D-loop formation. Two other mutants, *rad54 N616S* and *rad54 D442Y*, were not sensitive to genotoxic agents and behaved like the wild type allele in homologous recombination assays. Consistent with the mild phenotype associated with the *rad54 N616S* allele, its encoded protein was similar to wild type Rad54 protein in biochemical attributes. Because dysfunctional homologous recombination gives rise to genome instability, our results are consistent with the premise that tumor-associated mutations in hRad54 and Rad54B could contribute to the tumor phenotype or enhance the genome instability seen in tumor cells.

In *Saccharomyces cerevisiae*, homologous recombination (HR)[1] represents an important means for the repair of DNA double strand breaks and other types of DNA damage and also for restarting stalled replication forks (1–5). HR depends on the use of a homologous DNA molecule as a template to eliminate double strand breaks and other lesions mostly with high fidelity. Impaired HR results in genetic instability and sensitivity to genotoxic agents (6) and can lead to cancer in humans (7).

Double strand break repair by HR is dependent on genes of the *RAD52* epistasis group, *RAD50*, *RAD51*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RDH54/TID1*, *MRE11*, and *XRS2*, in which structure and function have been highly conserved (6). Before repair can occur, the ends of double strand breaks are processed by nucleolytic resection of the 5′ ends of the breaks to yield ssDNA, which is then bound by Rad51 to form a helical nucleoprotein filament often referred to as the presynaptic filament. Nucleation of Rad51 onto the ssDNA is facilitated by recombination mediators, including Rad52, the Rad55-Rad57 complex, and Rad54 (6, 8). Once assembled, the presynaptic filament mediates a search for homology in the homologous dsDNA partner and forms a DNA joint molecule (called D-loop) with the latter. The length of the nascent DNA joint molecule is extended by DNA branch migration (9), and the D-loop is resolved by one of several pathways that yields recombinants with or without associated crossovers (6).

The *RAD54* gene encodes a dsDNA-dependent ATPase of the Swi2/Snf2 family (10). Rad54 protein physically interacts with Rad51 protein (11–13) and binds ssDNA preferentially. Rad54 utilizes the free energy from ATP hydrolysis to translocate on dsDNA and induces dynamic topological changes in the DNA (8, 14, 15). The ATPase and DNA supercoiling activities of Rad54 are stimulated by Rad51 (15, 16). Rad54 protein appears to play a role in different steps of the HR process. Rad54 promotes assembly of the presynaptic filament apparently by loading Rad51 onto ssDNA and enhancing filament stability (17, 18). Although Rad51 has only a weak ability to form the D-loop, the inclusion of Rad54 renders D-loop formation highly robust (11, 15, 19, 20). The negative supercoils produced by Rad54 result in transient opening of the DNA strands (15), which may aid in strand invasion by the presynaptic filament in the D-loop reaction. Rad54 also stimulates DNA branch migration by severalfold (21) and cooperates with the presynaptic filament in chromatin remodeling (22–24). In addition, Rad54 can dissociate Rad51 from duplex DNA, an activity believed to be germane for Rad51 recycling and the promotion of DNA repair synthesis during recombination (25).

The protein encoded by *RDH54/TID1* is structurally related to Rad54 protein. Rdh54/Tid1 protein is important for meiotic recombination but has a relatively minor role in mitotic recombination (26, 27). Rdh54/Tid1 also possesses a dsDNA-activated ATPase activity via its dsDNA, binds Rad51, translocates on and supercoils dsDNA, and greatly stimulates D-loop

* This work was supported by United States Public Health Service Research Grants GM53738 (to H. L. K.) and GM57814 (to P. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HR, homologous recombination; ssDNA, single strand DNA; dsDNA, double strand DNA; MMS, methyl methanesulfonate; HU, hydroxyurea; BSA, bovine serum albumin; SC, synthetic complete; YEPD, yeast extract peptone dextrose.

formation (8, 28). It remains to be seen whether or not Rdh54/Tids affects presynaptic filament assembly, stimulates DNA branch migration, functions in chromatin remodeling, and removes Rad51 from duplex DNA.

Two Rad54 homologous proteins have been found in different eukaryotic species (29). The human hRad54 protein has 68% similarity with ScRad54 protein. A second human homologue, *RAD54B*, was described subsequently (30). The NH_2-terminal region of *RAD54B* shares homology with yeast *RDH54*. *In vitro* experiments show a dsDNA-dependent ATPase in hRad54 and that hRad54 translocates on dsDNA (14, 31) and promotes transient separation of the strands in duplex DNA (31). Interaction with hRad51 stimulates the ATPase activity, DNA supercoiling, and the DNA strand opening activities of hRad54. hRad51 and hRad54 cooperate in the formation of D-loops (31). Except for the demonstration of a dsDNA-dependent ATPase activity (32), the Rad54B protein remains poorly characterized.

In vertebrate cells, interference with HR has a strong impact on viability and genomic stability. For instance, cells with hypomorphic mutations in the *RAD51* gene are genetically unstable and sensitive to DNA-damaging agents, and disruption of the *RAD51* gene is lethal (33, 34). The importance of HR in cancer avoidance is best appreciated in studies of the breast tumor suppressor genes *BRCA1* and *BRCA2*. Cells with mutations in *BRCA1* and *BRCA2* are impaired for HR and show a high rate of chromosome aberration, sensitivity to DNA-damaging agents, and elevated mutation rates (35–42). BRCA2 binds Rad51 through a series of BRC repeats and is thought to promote the assembly of the presynaptic filament (40–42).

As expected, Rad54 and Rad54B both function in HR in mammalian cells. Inactivation of the mouse *RAD54* gene causes a marked sensitivity to DNA-damaging agents in embryonic stem cells, but somatic cells deleted for Rad54 show only a slight increase in radiosensitivity (43–46). Inactivation of the *RAD54B* gene in a colon cancer cell line resulted in a severe reduction of targeted integration frequency, whereas sensitivity to DNA-damaging agents and sister chromatid exchange was not significantly affected (47). hRad54 protein colocalizes with hRad51 and BRCA1 in the nucleus (48, 49), and significant colocalization of Rad54B with hRad51 and BRCA1 has also been observed (50). Interestingly, several point mutations in conserved regions of the *hRAD54* and *RAD54B* genes have been found in primary tumors (30, 51), but the functional consequences of these mutations on gene function and their causal relationship to the tumor phenotype is not known. To ascertain the possible effects of the tumor-associated *hRAD54* and *RAD54B* mutations on protein functions, we introduced these mutations into the homologous sites in the *S. cerevisiae RAD54* gene. We found that two mutations, *rad54 N616S* and *rad54 D442Y*, engendered little or no phenotype but that another mutation, *rad54 G484R,* behaved similarly to the *rad54*Δ null allele. The severe phenotype seen with the *rad54 G484R* allele was because of an inability of the mutant protein to hydrolyze ATP. The implications of our results are discussed.

EXPERIMENTAL PROCEDURES

Media, Growth Condition, and Genetic Analysis—Standard media were prepared as described (52). All strains were grown at 30 °C. Genetic analysis was performed according to standard procedure (52).

Construction of rad54 Mutants—Mutations were introduced into the *S. cerevisiae RAD54* gene using the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenic PCR primers were used in PCR reactions with the *RAD54* gene cloned into the pUC18 vector as a template. Mutation confirmation was performed by restriction enzyme digestion, which specifically recognizes each change, and by DNA sequencing to ensure that no other mutations were introduced inadvertently. For genetic analyses, pRS306 (*URA3*, integrating) derivatives containing the *rad54 D442Y*, *rad54 G484R*, or *rad54 N616S* allele were linearized by HindIII, purified, and then used for transforming wild type yeast strain HKY579-1A. Chromosomal integrants were selected on SC-ura media. After 2 days of growth on YEPD, the integrants were transferred to 5-fluoroorotic acid-containing media. 5-Fluoroorotic acid-resistant colonies were analyzed for the presence of the *RAD54* mutations by PCR analysis of the genomic *RAD54* gene, using the restriction enzymes diagnostic for each mutation and DNA sequencing of the PCR products.

For protein purification, the *rad54 G484R* and *rad54 N616S* alleles were cloned into pPM231.6His (*2μ, LEU2-d, GAL-PGK-6His*) to add a six-histidine sequence at the NH_2-terminal end of the *rad54*-encoded products and place the histidine-tagged mutant genes under the control of the galactose-inducible *GAL-PGK* promoter for expression in yeast cells (11). Plasmids pR54.G484R.1 (*2μ, LEU2-d, GAL-PGK-6His-rad54 G484R*) and pR54.N616S.1 (*2μ, LEU2-d, GAL-PGK-6His-rad54 N616S*) were transformed into the protease-deficient strain BJ5464.

Yeast Strains—Yeast strains for genetic studies were all derived from the W303 *RAD5* strain and have the basic genotype *ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*. Strains with point mutations in the *RAD54* gene were crossed with strains HKY660-2B *MAT***a** *leu2-rI*::*URA3*::*leu2-bsteII*, HKY661-4D *MAT***a** *rdh54*::*HIS3 leu2-rI*::*URA3*::*leu2-bsteII*, HKY590-6D *MAT***a** *srs2*::*HIS3*, HKY885 *MAT***a** *pol30-50*, and MSY127-5A *MAT***a** *top3*::*HIS3*. For the chromosome-loss assay, we constructed diploids from a set of strains with *ade2-1 can1-100 hom3-10* (or *ADE2 CAN1 HOM3*) in addition to the *RAD54* mutations. Sources of the markers were strains HKY1025-47D *MAT***a** *CAN1 ADE2 HOM3* and HKY1026-8C *MAT***a** *can-100 ade2-1 hom3-10*. The protease-deficient strain BJ5464 used in protein purification has the genotype *MAT***a**, *ura3-52*, *trp-1*, *leu2*Δ*1*, *his3*Δ*200*, *pep4*::*HIS3*, *prb*Δ*1.6R*.

DNA Substrates—ϕX 174 viral (+) strand DNA was purchased from New England Biolabs, and the ϕX-replicative form I DNA (~90% supercoiled) was from Invitrogen. Relaxation of the ϕX-replicative form I DNA by calf thymus topoisomerase I was carried out as described previously (19). pBluescript form I dsDNA was prepared using standard methods (53). For the DNA binding experiments, the 83-mer oligonucleotide (Oligo 3) (19) with the sequence 5′-TTG ATA AGA GGT CAT TTT TGC GGA TGG CTT AGA GCT TAA TTG CTG AAT CTG GTG CTG TAG CTC AAC ATG TTT TAA ATA TGC AA-3′ was 5′ end-labeled with [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega). The unincorporated nucleotide was removed with a Spin 30 column (Bio-Rad), and the radiolabeled oligo was annealed to its exact complement. The resulting duplex was purified from a 10% polyacrylamide gel by overnight diffusion at 4 °C into TAE buffer (40 mM Tris acetate, pH 7.5, 0.5 mM EDTA) (19). The 90-mer oligonucleotide D1 used in the D-loop reaction is complementary to pBluescript SK DNA from positions 1932 to 2022 and has the sequence 5′-AAA TCA ATC TAA AGT ATA TAT GAG TAA ACT TGG TCT GAC AGT TAC CAA TGC TTA ATC AGT GAG GCA CCT ATC TCA GCG ATC TGT CTA TTT-3′. The oligonucleotide was 5′ end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Biosciences) and then purified using the MERmaid spin kit (Bio101). All the DNA substrates were stored in TE buffer (10 mM Tris-HCl, pH 7.0, 0.5 mM EDTA).

Determination of Intrachromosomal Recombination Rates—Recombination rates were determined as described previously (54). Spore segregants from crosses of the *rad54* mutants with the recombination reporter strains were used. Nine colonies from each spore segregant were used for each fluctuation test, and three to six fluctuation tests were performed for each *rad54* mutant.

MMS and HU Sensitivity—To determine methyl methanesulfonate (MMS) and hydroxyurea (HU) sensitivity of the *rad54* mutants, cells were grown in liquid YEPD to saturation. The cells were then diluted, and 4-μl aliquots were spotted onto fresh YEPD plates containing 0.016% MMS or 200 mM HU.

For UV sensitivity, cells from overnight cultures were diluted, and 4-μl aliquots were spotted onto fresh YEPD plates and exposed to UV irradiation. After exposure, the plates were kept in the dark to avoid photoreactivation.

Determination of Chromosome Loss Rates in Diploids—Rates of chromosome loss were determined as described previously (55). Fresh zygotes were isolated for each genotype. After growth for 3–4 days at 30 °C on YEPD medium, zygotic colonies were resuspended in water. Appropriate dilutions were applied onto SC plates for measurement of the total number of colony-forming units and onto SC plates plus canavanine to select for canavanine-resistant mitotic segregants. These were then replica-plated to SC plates lacking threonine. Colonies that grew only on SC plus canavanine plates, Can^r Hom$^-$, were counted to determine the chromosome loss rates. Fluctuation tests were conducted

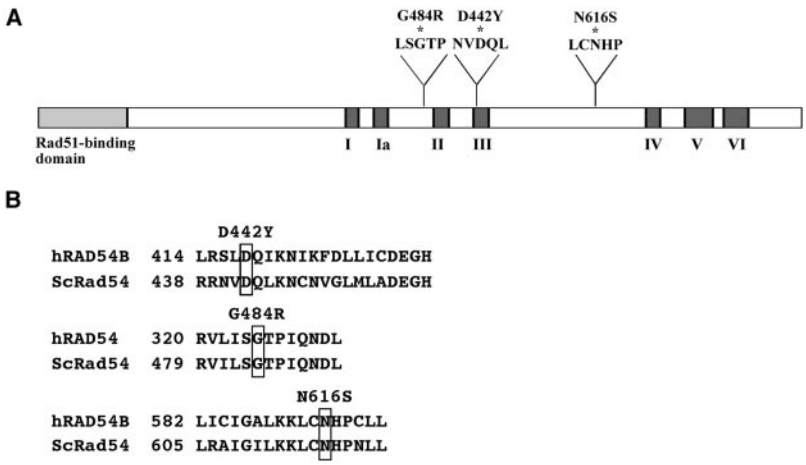

FIG. 1. **Schematic representation of the domain structure of the *S. cerevisiae* gene *RAD54* and position of the point mutations.** *A*, the conserved helicase domains I–VI are shown as well as the Rad51 binding domain. Positions of the mutations D442Y, G484R, and N616S relative to the helicase domains are shown. *B*, alignment of the hRad54B and hRad54 amino acid domains with the ScRad54 domains, showing that all amino acid changes are within conserved residues and conserved domains of the proteins.

according to the median method (56). These tests were repeated three to five times for each genotype.

Protein Expression and Purification—The rad54 N616S and rad54 G484R mutant proteins were purified to near homogeneity from cells of the BJ5464 strain harboring pR54.G484R.1 or pR54.N616S.1 using the procedure described for wild type Rad54 protein (11). The two mutant rad54 proteins behaved like the wild type protein in all of the fractionation steps. Rad51 was overexpressed and purified to near homogeneity from yeast as described by Sung (57). *Escherichia coli* topoisomerase I was purified to near homogeneity from *E. coli* JM103 (Stratagene) cells transformed with pJW312 as described by Lynn *et al.* (58). The concentration of purified Rad54, rad54 G484R, rad54 N616S, and *E. coli* topoisomerase I was determined by densitometric scanning of 8% SDS-polyacrylamide gels with multiple loadings of these proteins against known quantities of bovine serum albumin (BSA) and ovalbumin. The concentration of purified Rad51 was determined using the extinction coefficient 1.29×10^4 (59).

ATPase Assay—The indicated amounts of Rad54, rad54 G484R, and rad54 N616S proteins were incubated with ϕX174-replicative form I DNA (30 μM base pairs) in 10 μl of buffer A (30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, and 100 μg/ml BSA) and 1.5 mM [γ-^{32}P]ATP (Amersham Biosciences) for the indicated times at 23 °C. The amount of ATP hydrolysis was determined by thin layer chromatography as described by Petukhova *et al.* (11). To examine the effect of Rad51 on ATP hydrolysis by Rad54 and the rad54 mutants, Rad51 (360 nM) was premixed with Rad54, rad54 G484R, and rad54 N616S (50 nM each) at 0 °C for 15 min prior to the addition of DNA and incubation at 23 °C.

DNA Mobility Shift—The indicated amounts (40–200 nM) of Rad54, rad54 G484R, and rad54 N616S and the ^{32}P-labeled 83-mer duplex substrate (1.5 μM nucleotides) were incubated for 8 min at 23 °C in 10 μl of buffer A with 2.5 mM ATP and an ATP-regenerating system consisting of 10 mM creatine phosphate and 30 μg/ml creatine kinase. After adding 2 μl of loading buffer (30 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% orange G, 50% glycerol), the reaction mixtures were analyzed in 9% native polyacrylamide gels run in TAE buffer at 4 °C. The gels were dried and subject to analysis in a Personal FX phosphorimaging device employing the Quantity One software (Bio-Rad).

Rad51 and Rad54 Complex Formation—Rad51 and BSA were covalently conjugated to Affi-Gel 15 beads (Bio-Rad) to yield matrices containing 3 mg/ml Rad51 and 12 mg/ml BSA as described by Petukhova *et al.* (11). To examine binding of Rad54, rad54 G484R, and rad54 N616S to the affinity beads, these proteins (4 μg each) were gently mixed with 6 μl of Affi-Rad51 or Affi-BSA beads every 3 min for 45 min at 4 °C in 30 μl of binding buffer (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.01% Nonidet P-40, and 0.5 mM dithiothreitol) containing 150 mM KCl. The beads were collected by centrifugation, and the supernatant was removed. After being washed twice with 100 μl of binding buffer with 300 mM KCl, the Affi-Rad51 and Affi-BSA beads were treated with 30 μl of 3% SDS at 37 °C for 10 min to elute the bound Rad54 or mutant rad54 protein. The supernatant containing unbound Rad54 or mutant rad54 protein (10 μl), the two washes (15 μl), and the SDS eluate (10 μl) were subject to SDS-PAGE in an 8% gel to determine their content of Rad54 or mutant rad54 protein.

D-loop Reaction—The radiolabeled oligonucleotide D1 (3.6 μM nucleotides) was incubated with Rad51 (1.5 μM) in 10.5 μl of buffer A and an ATP-regenerating system consisting of 20 mM creatine phosphate and 30 μg/ml creatine kinase for 5 min at 37 °C followed by the incorporation of the indicated amounts (80–400 nM) of Rad54, rad54 G484R, or rad54 N616S in 1 μl storage buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 300 mM KCl) and a 2-min incubation at 23 °C. The reaction was initiated by adding the pBluescript-replicative form I DNA (35 μM base pairs) in 1 μl TE (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA). The reaction mixtures were incubated at 23 °C for 4 min and processed for electrophoresis in 0.9% agarose gels in TAE buffer at 23 °C as described previously (20). The gels were dried, and the radiolabeled DNA species were visualized and quantified in the phosphorimaging device. The percent D-loop refers to the quantity of the replicative form I substrate that had been converted into D-loop.

Topoisomerase I-linked DNA Supercoiling Assay—The indicated amounts of Rad54, rad54 G484R, and rad54 N616S were incubated with topologically relaxed ϕX 174 DNA (18.5 μM base pairs) for 3 min at 23 °C in 9.5 μl of buffer R (35 mM Tris-HCl, pH 7.6, 2.5 mM ATP, 3 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 1 mM dithiothreitol, and an ATP regenerating system consisting of 20 mM creatine phosphate and 30 μg/ml creatine kinase) with 50 mM KCl followed by the addition of 150 ng of *E. coli* topoisomerase I in 0.5 μl of storage buffer. The reactions were incubated at 23 °C for 10 min and processed for agarose gel electrophoresis as described previously (19). To examine the effect of Rad51, it was mixed with Rad54, rad54 G484R, and rad54 N616S at 4 °C for 20 min prior to the addition of the DNA substrate and incubation with topoisomerase.

RESULTS AND DISCUSSION

Phenotypes of the rad54D442Y, rad54 N616S, and rad54 G484R Mutants—The human cancer cell lines Kco15, Ly6, and Br7 harbor mutations in *RAD54B* and *hRAD54* genes at positions that are highly conserved among Rad54-homologous proteins. In Kco15, in a region close to helicase domain II, a conserved Asp residue at codon 418 of Rad54B (equivalent to Asp-442 in yeast Rad54) is mutated to Tyr (30) (Fig. 1). In Ly6, the Asn at codon 593 (equivalent to Asn-616 in yeast Rad54), a residue within a conserved region between helicase motifs III and IV, is mutated to Ser in Rad54B (30) (Fig. 1). In Br7, in the helicase motif III of hRad54, the conserved Gly residue at codon 325 (equivalent to Gly-484 in yeast Rad54) is mutated to Arg (51) (Fig. 1). We introduced point mutations into the *S. cerevisiae RAD54* gene corresponding to the aforementioned mutations found in the human cancer cell lines (Fig. 1).

Strains carrying the *rad54* mutations (*D442Y, N616S*, and *G484R*) were examined for sensitivity to MMS, HU, and UV irradiation. The *rad54 G484R* mutant showed a degree of sensitivity to all three DNA-damaging agents very similar to that seen with the deletion mutant *rad54*Δ (Fig. 2). In crosses of *rad54 G484R* by *RAD54*, the *rad54 G484R* spore segregants showed slower mitotic growth compared with the *RAD54* strain. In contrast, *rad54 D442Y* and *rad54 N616S* were not hypersensitive to MMS, HU, or UV irradiation (Fig. 2), and they did not exhibit any growth defect.

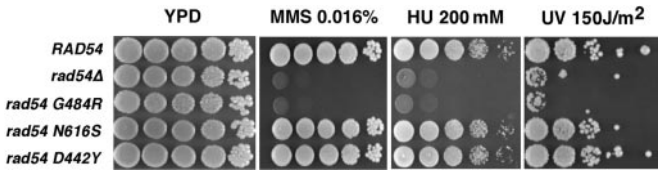

FIG. 2. **DNA damage sensitivities of the *rad54* mutants.** Cells of each genotype were grown overnight in YEPD, the culture was serially diluted 10-fold, and 4 μl from each dilution was spotted onto YEPD plates containing 0.016% MMS, 200 mM HU, or without any addition (*YPD*). The cells were also spotted onto YEPD plates and exposed to UV irradiation at a dose of 150 J/m^2 exposure. The plates were photographed after 3 days of growth at 30 °C.

Homozygous *rad54* mutant diploids demonstrated differences in spore viability, a reflection of meiotic recombination success. The *rad54 G484R* diploid has impaired spore viability of 63% compared with spore viability of 93% for *RAD54* and 68% for the *rad54*Δ null allele diploid. The *rad54 D442Y* and *rad54 N616S* homozygous mutant diploids had spore viability of 97 and 98%, respectively.

Effect of the rad54 Mutations on Intrachromosomal Recombination—Intrachromosomal gene conversion is strongly reduced in the *rad54*Δ mutant (19). To determine the effect of the *rad54* mutations on intrachromosomal gene conversion, we tested recombination in haploid *rad54 N616S, rad54 D442Y*, and *rad54 G484R* strains using the *leu2-rI*::*URA3*::*leu2-bsteII* direct repeat. We measured gene conversion and deletion events by determining the rates of the Leu$^+$ Ura$^+$ colonies and 5-fluoroorotic acid-resistant (Ura$^-$) events, respectively. As shown in Fig. 3, the *rad54 G484R* mutant has a reduced rate of gene conversion and an increased gene deletion rate compared with wild type, and the magnitude of the effects is very similar to that observed with the null mutant *rad54*Δ. Intrachromosomal gene conversion and deletion rates in the *rad54 D442Y* and *rad54 N616S* mutants do not differ from the wild type strain.

Chromosome Loss in rad54 Mutants—Chromosome stability is strongly impaired in the *rad54*Δ deletion mutant (55). We examined the effect of the three *rad54* point mutations on chromosome stability in homozygous mutant diploids. Chromosome stability was measured by a genetic assay for loss of one copy of chromosome V. One chromosome V homologue was marked with *hom3-10* and *can1-100*, whereas the other homologue harbored the wild type alleles of these genes. Can^r Hom$^-$ segregants were classified as chromosome loss events (55).

The chromosome loss rate in the *rad54 G484R* diploid was elevated to the same level as that observed in the *rad54*Δ diploid, an increase of about 40-fold over the *RAD54* rate (Fig. 4). The *rad54 N616S* and *rad54 D442Y* mutant diploids had a wild type rate of chromosome loss, again suggesting that these mutations do not significantly affect the function of Rad54 (Fig. 4).

Genetic Interactions—To further assess the phenotype of the *rad54 G484R* mutant, we studied the genetic interactions of this allele with mutations known to be lethal in a *rad54*Δ background. The *srs2*Δ mutation is lethal in a *rad54*Δ strain (60). Genetic analysis of the crosses between *rad54 G484R* and *srs2*Δ shows synthetic lethality as well.

Synthetic lethality of the *rad54 G484R* and *rad54*Δ mutations with the *pol30-52* mutation in the *POL30* gene, which encodes the DNA polymerase processivity clamp PCNA, was also found. Mutations in the *RAD54* gene can suppress the slow growth and hyper-recombination phenotypes of the *top3* mutant (61). Our genetic analysis showed that the *rad54 G484R* mutation could suppress the slow growth of *top3*Δ. Recombination rate determinations revealed that the *rad54 G484R* mutation resulted in a 20-fold decrease in gene conversion of the *top3*Δ mutant compared with the *RAD54 top3*Δ strain. In contrast, the *rad54 N616S* and *rad54 D442Y* mutants are viable in combination with *srs2*Δ or *pol30-52* and do not show any effect on the hyper-recombination phenotype of the *top3*Δ mutant.

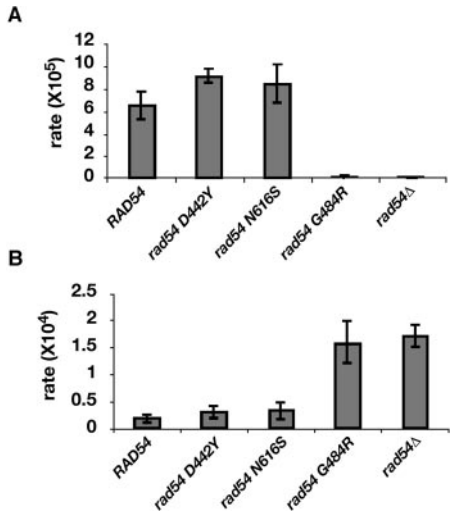

FIG. 3. **Effects of the *rad54 D442Y, rad54 G484R*, and *rad54 N616S* mutations on homologous recombination.** Rates of intrachromosomal gene conversion and deletion in the haploid mutants were determined using the direct repeat *leu2-rI*::*URA3*::*leu2-bsteII*. For rate determinations, three spore segregants from crosses of the *rad54* mutants with HKY660-2B were used. *A*, rates of gene conversion were determined as rates of Leu$^+$ Ura$^+$ mitotic segregants. *B*, deletion rates were determined as rates of 5-fluoroorotic acid-resistant mitotic segregants. The mean of the rates, from three to six independent rate determinations, is presented with the standard deviation.

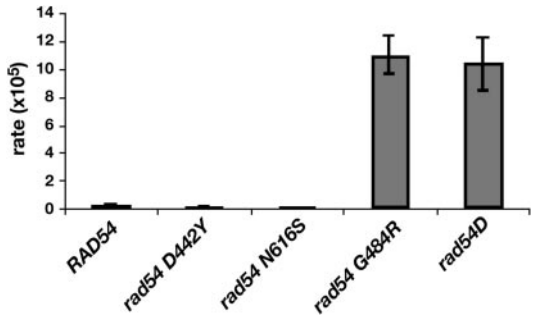

FIG. 4. **Rates of chromosome loss in *rad54* diploids.** Determination of chromosome loss rates in mutant and wild type diploids was performed as described under "Experimental Procedures." All diploids were isogenic to the W303 parental strain. Chromosome loss rates were determined three times for each genotype. The mean and standard deviation of these rates is shown.

As detailed above, the *rad54 N616S* and *rad54 D442Y* mutants did not exhibit increased sensitivity to genotoxic agents and had wild type levels of mitotic gene conversion and chromosome loss. However, the *rad54 N616S* mutation was not completely benign. We observed that diploid strains hemizygous for the *rad54 N616S* allele (*rad54 N616S*/*rad54*Δ) had increased MMS sensitivity compared with the homozygous *rad54 N616S* diploid (*rad54 N616S*/*rad54 N616S*). Hemizygous *RAD54* strains (*RAD54*/*rad54*Δ) did not show any increased MMS sensitivity.

Further evidence of a subtle functional impairment in the *rad54 N616S* mutant was revealed by double mutant studies of HU and MMS sensitivity. The double mutants *rad54 N616S rdh54*Δ and *rad54 N616S srs2*Δ had a slight enhancement of MMS and HU sensitivity compared with the single mutants (Fig. 5). We did not, however, find any change in rates of gene conversion and deletion formation in these double mutants

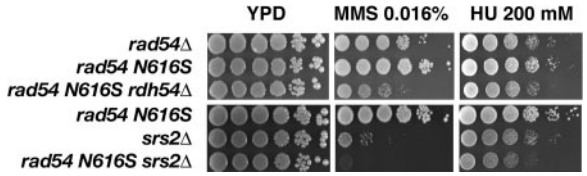

FIG. 5. **Enhancement of MMS and HU sensitivity in *rad54 N616S rdh54*Δ and *rad54 N616S srs2*Δ double mutants.** MMS and HU sensitivities were determined as described in Fig. 2. Double mutants were generated in crosses of *rad54 N616S* mutant with HKY661-4D and HKY590-6D strains. The plates were photographed after 3 days of growth at 30 °C on YEPD plates containing 0.016% MMS or 200 mM HU or without any addition (*YPD*).

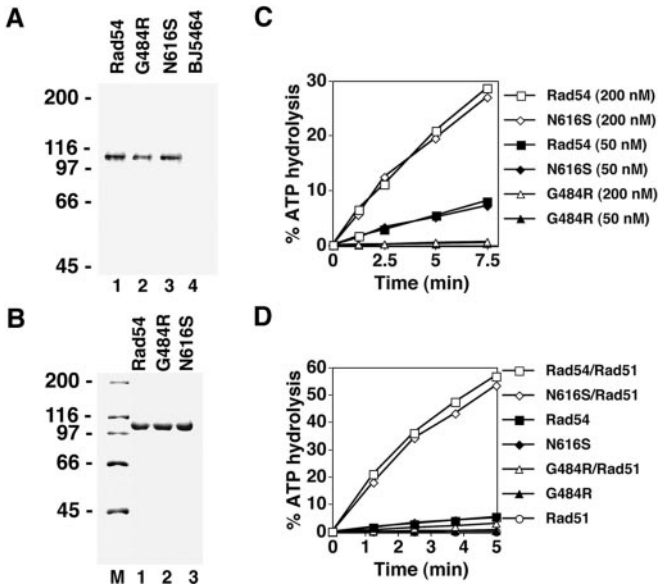

FIG. 6. **ATP hydrolysis by Rad54, rad54 G484R, and rad54 N616S.** *A*, immunoblot analysis of extract from yeast strain BJ5464 harboring the empty protein expression vector pPM231.6His (*lane 4*) and plasmids pR54.1, pR54.G484R.1, and pR54.N616S.1 that express Rad54 (*lane 1*), rad54 G484R (*lane 2*), and rad54 N616S (*lane 3*), respectively, using anti-Rad54 antibodies as described by Petukhova *et al.* (11). *B*, purified Rad54, rad54 G484R (G484R), and rad54 N616S (N616S) proteins, 1.8 μg of each, were run beside size markers (*M*) in an 8% denaturing polyacrylamide gel and then stained with Coomassie Blue. *C*, time course of ATP hydrolysis by Rad54, rad54 G484R, and rad54 N616S. Rad54, rad54 G484R, and rad54 N616S (all 50 and 200 nM) were incubated with 1.5 mM [γ-^{32}P]ATP and ϕX-replicative form I DNA (18.5 μM base pairs) for the indicated times. *D*, to examine the effect of Rad51 on ATPase hydrolysis by the mutant rad54 proteins, 50 nM Rad54, rad54 G484R, and rad54 N616S were incubated with radiolabeled ATP, ϕX DNA, and 200 nM Rad51 or without Rad51 for the indicated times. Rad51 was also incubated with the radiolabeled ATP and ϕX-replicative DNA for the indicated times.

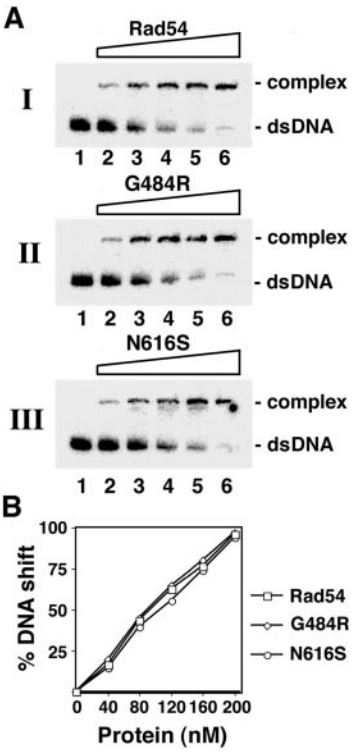

FIG. 7. **DNA binding by Rad54, rad54 G484R, and rad54 N616S.** *A*, ^{32}P-labeled 83-mer duplex (1.5 μM nucleotides) was incubated with Rad54 protein (*panel I*, *lanes 2–6*), rad54 G484R (*panel II*, *lanes 2–6*), and rad54 N616S (*panel III*, *lanes 2–6*) all at 40, 80, 120, 160, and 200 nM. The reaction mixtures were analyzed in nondenaturing polyacrylamide gels, which were dried and subjected to phosphorimaging analysis. No protein was added in *lanes 1*. *B*, graphical representation of the results in *A*.

(data not shown). Surprisingly, the mitotic recombination rate of the *rad54 N616S srs2*Δ double mutant showed an increase of 15-fold over the *srs2*Δ rate. The double mutant colonies grew slowly with uneven edges featuring lethal sectors.

rad54 G484R, but Not rad54 N616S, Is Impaired for ATP Hydrolysis—To examine the effects of the tumor-associated hRad54 and Rad54B mutations on the biochemical activities of Rad54, the rad54 G484R and rad54 N616S proteins were overexpressed in yeast cells (Fig. 6*A*) and purified to near homogeneity (Fig. 6*B*) by following the procedures devised for the purification of the wild type protein (19). We used thin layer chromatography to examine the ability of the rad54 G484R and rad54 N616S mutant proteins to hydrolyze ATP in the presence of dsDNA. The results, as summarized in Fig. 6*C*, indicated that whereas rad54 N616S is just as proficient as the wild type protein in ATP hydrolysis, the rad54 G484R mutant protein possesses little or no ATPase activity (Fig. 6*C*).

Published studies have shown that through a specific protein-protein interaction, Rad51 greatly stimulates the ATPase of Rad54 (15, 16). We examined whether Rad51 can enhance the ATPase activity of these mutant proteins. As shown in Fig. 6*D*, Rad51 greatly enhanced the ATPase activity of wild type Rad54 and rad54 N616S. In contrast, the addition of Rad51 did not enable rad54 G484R to hydrolyze ATP.

rad54 G484R and rad54 N616S Bind DNA and Rad51—The data above show a pronounced defect of the rad54 G484R mutant in ATP hydrolysis. Because the Rad54 ATPase activity requires dsDNA as a cofactor, it was of considerable interest to examine whether the noted defect associated with the rad54 G484R mutant was due to an inability to bind DNA. DNA mobility shift experiments were performed to address this point. Rad54, rad54 G484R, and rad54 N616S were incubated with a ^{32}P-labeled 83-mer duplex, and the levels of nucleoprotein complex formed were compared. Importantly, the results from this experiment showed that rad54 G484R is just as proficient in DNA binding as the rad54 N616S and wild type proteins (Fig. 7).

Rad54 physically interacts with Rad51 *in vitro* and *in vivo* (11–13). To determine whether the mutant rad54 G484R and rad54 N616S proteins retain the ability to bind Rad51, purified rad54 G484R and rad54 N616S were mixed with Affi-gel 15 beads containing either Rad51 (Affi-Rad51) or bovine serum albumin (Affi-BSA). After washing with buffer, Rad54 and rad54 mutant proteins that remained bound to the Affi-beads were eluted by SDS and then analyzed by SDS-PAGE. As indicated from Fig. 8, as much of the rad54 G484R (*panel II*) and rad54 N616S (*panel III*) as the wild type (*panel I*) protein were found associated with the Affi-Rad51 beads. As ex-

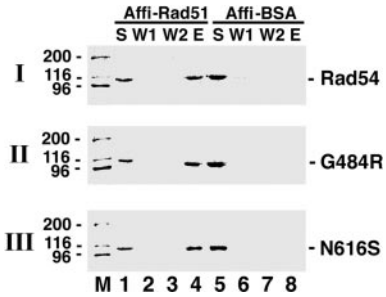

FIG. 8. **rad54 G484R and rad54 N616S mutant proteins physically interact with Rad51.** Purified Rad54, rad54 G484R, and rad54 N616S were mixed with Affi-Gel 15 beads containing either Rad51 (*Affi-Rad51*) or bovine serum albumin (*Affi-BSA*). The beads were collected by centrifugation, washed twice with buffer containing 300 mM KCl, and then eluted with SDS. The supernatant (*S*), first wash (*W1*), second wash (*W2*), and SDS-eluate (*E*) were run in an 8% denaturing polyacrylamide gel followed by staining with Coomassie Blue.

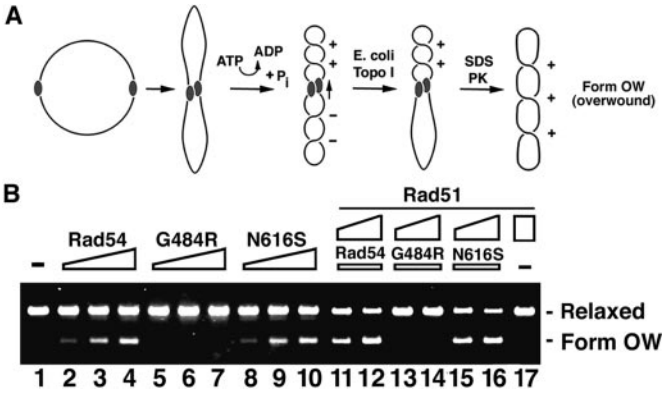

FIG. 9. **Rad54-mediated DNA supercoiling is inactivated by the G484R mutation.** *A*, schematic of the *E. coli* topoisomerase I-linked DNA supercoiling assay, as described by Ristic *et al.* (14) and Van Komen *et al.* (15). ATP hydrolysis fuels the translocation of a Rad54 oligomer on DNA, producing a positively supercoiled domain ahead of protein movement and a negatively supercoiled domain behind. The addition of *E. coli* topoisomerase I removes the negative supercoils, resulting in the formation of a positively supercoiled species called Form OW upon deproteinization. *B*, Rad54 protein (*lanes 2–4*), rad54 G484R (*lanes 5–7*), and rad54 N616S (*lanes 8–10*) (all at 45, 90, and 135 nM) were incubated with topologically relaxed DNA (9 μM base pairs) and *E. coli* topoisomerase I. To test the effect of Rad51, this protein at 100 and 300 nM was added to DNA supercoiling reactions that contained the lowest amount (45 nM) of Rad54 or rad54 mutant. In *lane 1*, relaxed DNA was incubated in buffer with topoisomerase but without any recombination protein. In *lane 17*, Rad51 (300 nM) was incubated with relaxed DNA and topoisomerase. All the reactions were carried out at 23 °C for 10 min. The reaction mixtures were deproteinized and then analyzed in a 0.9% agarose gel followed by staining with ethidium bromide.

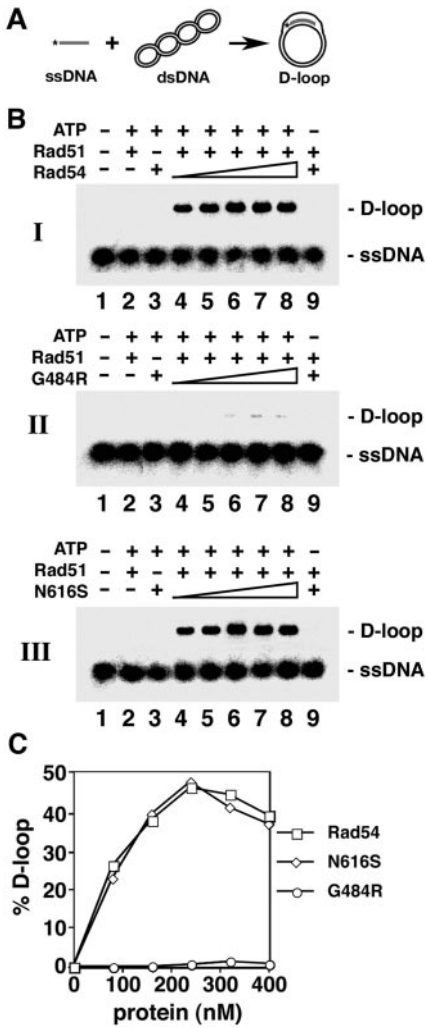

FIG. 10. **The rad54 G484R mutant has greatly attenuated activity in the D-loop reaction.** *A*, schematic of the D-loop reaction. Pairing of the radiolabeled 90-mer oligonucleotide with pBluescript form I DNA yields a D-loop. *B*, D-loop reactions with Rad51 only (*panels I*, *II*, and *III*, *lane 2*), Rad54 only (400 nM in *panel I*, *lane 3*), rad54 G484R only (400 nM in *panel II*, *lane 3*), rad54 N616S only (400 nM in *panel III*, *lane 3*), Rad51 and Rad54 (80, 160, 240, 320, and 400 nM in *panel I*, *lanes 4–8*), Rad51 and rad54 G484R (80, 160, 240, 320, and 400 nM in *panel II*, *lanes 4–8*), and Rad51 and rad54 G616S (80, 160, 240, 320, and 400 nM in *panel III*, *lanes 4–8*). Rad51 was at 1.5 μM, and the completed reaction mixtures were incubated for 4 min. In *lane 1* of each panel, the DNA substrates were incubated in buffer without any recombinant proteins. The reaction mixtures were deproteinized and resolved in 0.9% agarose gels, which were dried and subjected to phosphorimaging analysis. *C*, graphical representation of the results in *B*.

pected, neither wild type Rad54 nor the two rad54 mutants bound to the Affi-BSA beads (Fig. 8). Thus, the two rad54 mutants are as proficient as wild type Rad54 in complex formation with Rad51.

rad54 G484R, but Not rad54 N616S, Is Defective in DNA Supercoiling—Biochemical analyses and scanning force microscopy have indicated that the free energy derived from ATP hydrolysis fuels the translocation of Rad54 on duplex DNA. As Rad54 tracks on dsDNA, positive and negative supercoils that are equal in magnitude are generated, with the positive supercoils accumulating ahead of protein movement and the negative supercoils trailing it (8, 14, 15). The supercoiling activity of Rad54 is greatly stimulated via a specific interaction of Rad54 with the Rad51-ssDNA nucleoprotein filament. During recombination, the tracking of Rad54 on duplex DNA and the negative supercoils that accompany this motion are believed to enhance the rate at which the incoming duplex DNA can be sampled by the presynaptic filament for homology and to promote DNA joint formation upon location of DNA homology (9).

We have previously devised a topoisomerase I-linked assay to gauge the DNA supercoiling activity of Rad54 (15). Briefly, the negative supercoils generated by Rad54 are removed by *E. coli* topoisomerase I to result in the formation of a positively supercoiled species called Form OW (overwound), which is separated from the topologically relaxed input DNA by agarose gel electrophoresis ((15); Fig. 9*A*). We used this topoisomerase-linked assay to determine the ability of rad54 G484R and rad54 N616S to supercoil DNA. Fig. 9*B* shows that although rad54 N616S (*lanes 8–10*) was as capable of producing Form OW as the wild type protein (*lanes 2–4*), the rad54 G484R (*lanes 5–7*) mutant is devoid of this activity.

As with its ATPase activity, the DNA supercoiling activity of Rad54 is greatly stimulated via a specific interaction with Rad51 (15, 16). Consistent with data from the experiment that examined the Rad54 ATPase function (Fig. 6*D*), Rad51 enhanced the DNA supercoiling activity of rad54 N616S to the same extent as it did the wild type protein (Fig. 9*B*, *lanes 11* and *12*), but it did not impart DNA supercoiling activity to the rad54 G484R mutant (Fig. 9*B*, *lanes 13* and *14*).

Promotion of Rad51-mediated D-loop Formation—During the initial stage of homologous recombination, the Rad51-ssDNA nucleoprotein filament invades the homologous duplex donor to form a D-loop. The D-loop reaction can be studied by monitoring the incorporation of an oligonucleotide into a homologous supercoiled DNA molecule (Fig. 10*A*). Although Rad51, by itself, has only a modest ability to form the D-loop (11), the addition of Rad54 protein renders D-loop formation robust (11). Promotion of the D-loop reaction requires the ATPase activity of Rad54 (19). We examined the ability of rad54 G484R and rad54 N616S to enhance Rad51-mediated D-loop formation. To do this, Rad51 was incubated with the radiolabeled oligonucleotide before the homologous duplex (pBluescript-replicative form I DNA) and increasing amounts of Rad54, rad54 G484R, and rad54 N616S were incorporated. The wild type Rad54 and the rad54 N616S mutant were equally effective in the D-loop reaction, whereas only a negligible amount of D-loop was detected when rad54 G484R was used (Fig. 10, *B* and *C*).

Implications of Our Genetic and Biochemical Results—We have constructed several mutations in yeast *RAD54* to test the genetic and biochemical consequences of *hRAD54* and *hRAD54B* mutations found in human tumors. Of the three mutations tested, *rad54 G484R* showed great impairment of Rad54 activity both in genetic and biochemical assays. Whether this is related to the fact that the human mutation in this case, Br7, was in the *hRAD54* gene (and not the *hRAD54B* gene) is not known, as we have not tested yeast *RDH54* with a similar mutation. Nonetheless, our studies have revealed that the Br7 cell line most likely has no hRad54 activity and that the helicase III domain is essential for hRad54 activity.

In all of the genetic and biochemical aspects examined, the *rad54 G484R* mutant was as defective as the null allele deletion mutant. The *in vivo* phenotypes of reduced recombination and increased DNA damage sensitivities and genomic instability can be correlated with the loss of ATP hydrolysis activity and, consequently, DNA supercoiling, DNA strand opening, and Rad51-mediated D-loop formation. However, the rad54 G484R protein still retains the ability to bind DNA and Rad51 protein, demonstrating that nucleoprotein complex formation between Rad54 and DNA and association with Rad51 are not sufficient for DNA damage repair, DNA damage avoidance, and maintenance of genomic stability.

In contrast, the *rad54 D442Y* and *rad54 N616S* mutants that correspond to two tumor-associated mutations (D418Y and N593S, respectively) found in the *hRAD54B* gene (30) have no demonstrable deficiency in Rad54 protein activity and, accordingly, do not engender any discernible phenotype as single mutants. In double mutant studies, the *rad54 N616S* mutant showed an enhancement of DNA damage sensitivity. Overall, our genetic observations are consistent with the premise that the *rad54 N616S* mutant could have phenotypic consequences in certain mutant backgrounds.

In conclusion, our results suggest that the tumor-associated *hRAD54* mutation (G325R) noted in the Br7 cell line (51) probably enhances genomic instability in tumor cells. It is possible that this mutation accelerates the tumor phenotype by enhancing genomic instability in an already mutant cell line or may have a more causative role in tumorigenesis. Further experiments are required to test these possibilities.

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