Role of Human and Mouse Rad54

in DNA Recombination and Repair

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The back cover shows the redistribution of the mouse Rad54 and Rad51 proteins after treatment with ionizing radiation (for details see Chapter 7).

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Voor mijn ouders

Voor Monica

CONTENTS

LIST OF ABBREVIATIONS

ΔLK	change in linking number
AT	ataxia-telangiectasia
CHO	Chinese hamster ovary
CSB	Cockayne syndrome B
DNA-PK	DNA-dependent protein kinase
DNA-PK$_{CS}$	DNA dependent protein kinase catalytic subunit
ds	double-stranded
DSB	double-strand break
ELISA	enzyme linked immunosorbent assay
ES	embryonic stem
HA	hemagglutin
IR	ionizing radiation
MEF	mouse embryonic fibroblast
MMC	mitomycin C
MMS	methyl methanesulfonate
MN	micronuclei
NBS	Nijmegen breakage syndrome
PCE	polychromatic erythrocyte
PGK	phosphoglycerate kinase
RB	retinoblastoma
RPA	replication protein A
Scid	severe combined immuno deficiency
ss	single-stranded
SSB	single-strand DNA binding protein
TBP	TATA box-binding protein
TCR	T cell receptor

SCOPE OF THE THESIS

DNA double-strand breaks (DSBs) which can be induced by endogenously produced radicals or by ionizing radiation are among the most genotoxic DNA lesions. Repair of DSBs is of cardinal importance for the prevention of chromosomal fragmentation, translocations, and deletions. The genetic instability resulting from persistent or incorrectly repaired DSBs can eventually result in cancer. Therefore, to understand the biological consequences of exposure to ionizing radiation, insight into the mechanisms of DSB repair in mammalian cells is essential. The pace of identification of mammalian DSB repair genes has rapidly increased over the last few years. However, the functional analysis of the encoded proteins and the analysis of the role of the different DSB repair mechanisms in mammals are far from complete. This thesis describes the generation and phenotypic characterization of cells and mice, with a defect in one of the DSB repair genes, the *RAD54* recombinational DNA repair gene. Furthermore, the initial characterization and cellular behavior of the mammalian Rad54 protein is described. **Chapter 1** outlines the current knowledge on the role and molecular mechanisms of the multiple pathways that have evolved for the repair of DSBs. Our main findings concerning mammalian Rad54 at the protein and cellular level are discussed and integrated in the emerging picture of the DSB repair mechanisms in mammals. **Chapters 2** and **3** describe the isolation of mammalian *RAD54* genes and genomic characterization of the mouse *RAD54* gene. **Chapters 4** and **5** describe the generation and phenotypic characterization of *RAD54* knockout cells and mice. **Chapters 6** and **7** describe the characterization of the *in vitro* activities of the purified human Rad54 protein and the cellular behavior of the mouse Rad54 protein upon induction of DNA damage.

CHAPTER 1

Pathways of DNA double-strand break repair

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PATHWAYS OF DNA DOUBLE-STRAND BREAK REPAIR

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Among the most genotoxic DNA lesions generated by ionizing radiation are DNA double-strand breaks (DSBs). DSBs provide substantial threats to the genomic integrity of cells since they can result in chromosomal aberrations which, in turn, can lead to cell death or uncontrolled cell growth. Multiple pathways for repair of DSBs have evolved. The critical difference between the two main pathways, homologous recombination and DNA end-joining, is the fidelity of repair. While homologous recombination ensures accurate DSB repair, DNA end-joining does not. Results of initial experiments suggested that yeast cells use predominantly homologous recombination to repair DSBs, while mammalian cells mainly depend on DNA end-joining.

Here, we review results of recent experiments that revealed the functional conservation of both DSB repair pathways from fungi to humans. Given the potential difference in repair fidelity, an important challenge ahead is to determine the relative contribution of homologous recombination and DNA end-joining to maintenance of genomic integrity and ionizing radiation resistance in different mammalian cell types.

1 INTRODUCTION

DNA double-strand breaks (DSBs) are induced by endogenously produced radicals and ionizing radiation, which is often used in anti-cancer therapy. The accurate repair of DSBs is essential to prevent chromosomal fragmentation, translocations, and deletions. The persistence of chromosomal aberrations, resulting from incorrect DSB repair can lead to carcinogenesis through activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity. Therefore, insight into the molecular mechanisms of DSB repair is essential in order to understand the biological consequences of exposure to ionizing radiation.

Several mechanistically distinct DSB repair pathways have evolved, probably due to the extreme genotoxicity of DSBs. This review focuses on the mechanisms of two major DSB repair pathways. Figure 1 outlines the salient features of these two DSB repair pathways. The first, homologous recombination, requires extensive regions of homology and repairs DSBs accurately using the information on the undamaged sister-chromatid or homologous chromosome [1-4]. The second, DNA end-joining, uses no or extremely limited sequence homology to rejoin broken ends in a manner that need not to be error free [5, 6]. An additional, more specialized, DSB repair pathway is single-strand annealing. It can operate when the DSB occurs between or within directly repeated DNA sequences. DSB repair through single-strand annealing results in the loss of one of the repeats as well as the DNA segment between the repeats [7].

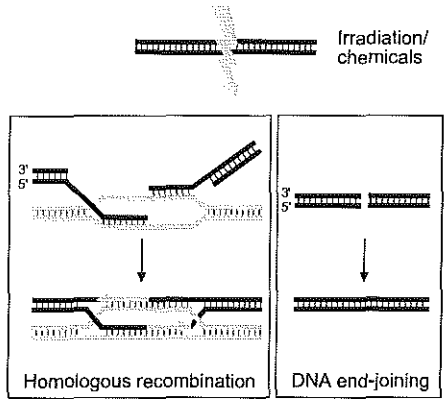

Figure 1. Pathways of DSB repair in eukaryotes. Two main pathways of DSB repair, homologous recombination and DNA end-joining, are schematically depicted. Symbols: thick lines, DNA strands; thin lines, base pairs; arrows in duplex DNA, newly synthesized DNA. Key events of homologous recombination include the processing of the broken ends to generate single-stranded DNA tails with 3' ends, the search for homologous duplex DNA and strand exchange, DNA synthesis to restore the lost information, branch migration of DNA crossovers (Holliday junctions) to extend heteroduplex DNA, and resolution of the Holliday junctions to yield two intact duplex DNAs. DNA end-joining probably involves processing of the ends by a nuclease and the subsequent ligation of the broken ends. A critical difference between the pathways is the fidelity of repair. While homologous recombination ensures accurate repair, DNA end-joining does not.

2 DSB REPAIR IN *ESCHERICHIA COLI*; THE RecA PATHWAY

The prokaryote *E. coli* has provided a paradigm for the involvement of homologous recombination in DSB repair. Therefore, we will briefly review the DSB repair process in *E. coli*. Genetic and biochemical analyses established that most of the homologous recombination in this organism is dependent on the RecA protein [8]. Homologous recombination can be initiated by a DSB that is processed to expose regions of single-stranded (ss) DNA as described below and schematically indicated in Figure 2. The ssDNA region is the substrate on which RecA protein binds cooperatively to form a presynaptic nucleoprotein filament containing three nucleotides per RecA promoter. The nucleoprotein filament plays a central role in homologous recombination. During synapsis, the filament mediates the search for homologous double-stranded (ds) DNA, followed by DNA strand exchange which results in the formation of a joint molecule between the ss- and dsDNAs (Figure 2B). Although RecA carries out central steps in homologous recombination, it relies on the action of DNA helicases and nucleases to generate its substrate and to process its product. In addition, regulatory proteins are needed for proper RecA nucleoprotein filament assembly and disassembly.

RecBCD, a multiprotein complex containing both helicase and nuclease activities, generates the substrate for RecA [9]. A very elegant mechanism involving a switch in the polarity of the RecBCD nuclease activity results in the production of ssDNA with a 3' end. The trigger of this switch is the sequence 5'-GCTGGTGG-3' which is known as a Chi site. RecBCD enters at a dsDNA end where its helicase activity initiates unwinding of the duplex. The nuclease activity of RecBCD

degrades the strand containing the 3' end. Upon encountering a properly oriented Chi site, the polarity of the nuclease activity switches [10]. Instead of degrading the strand containing the 3' end, the strand containing the 5' end is degraded. It is unclear how the nuclease polarity switch of RecBCD is brought about. However, the RecD subunit is clearly implicated in this process [9]. The result of the nuclease polarity switch at Chi is that continued unwinding by RecBCD produces a ssDNA tail with a 3' end 4 to 6 bases from the sequence 5'-GCTGGTGG-3' (see Figure 2A). Analysis of the complete *E. coli* genome sequence showed that Chi sites are embedded in GT-rich stretches of DNA [11]. Interestingly, the RecA protein binds with greater affinity to GT-rich stretches of DNA than to DNA rich in other dinucleotide sequences [12]. Thus, Chi sequences cause the RecBCD enzyme to produce a ssDNA tail with a 3' end in a sequence context that is favorable for RecA binding.

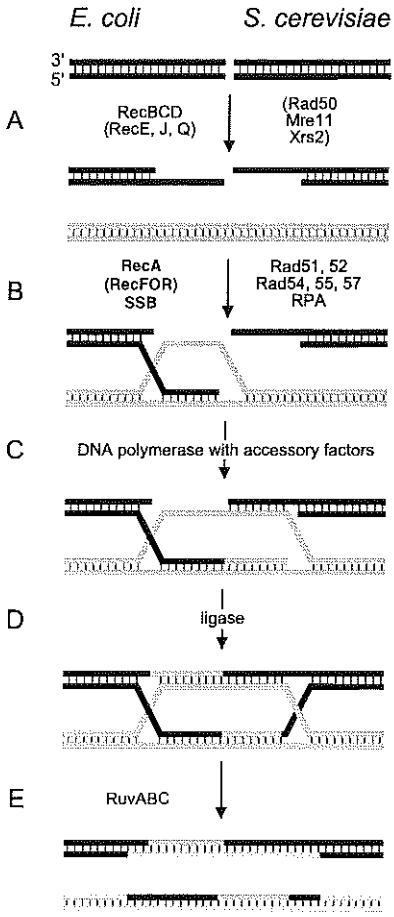

Figure 2. A model for DSB repair through homologous recombination. Details of the mechanism are discussed in the text [146-150]. **A.** Processing of the break by the combined action of helicases and/or nucleases results in the generation of ssDNA tails with a 3' overhang. **B.** The ssDNA tail is bound by a strand exchange protein to form a presynaptic nucleoprotein filament. This filament mediates the search for homologous dsDNA. Subsequent strand exchange by one of the filaments joins the recombination partners into a D-loop structure. **C.** The D-loop is enlarged by DNA synthesis and the second 3' end pairs with the displaced strand. **D.** Repair synthesis is initiated at the second 3' end to produce a joint in which the two DNA molecules are linked by two Holliday junctions. **E.** The Holliday junctions are resolved by a specific endonuclease resulting in release of recombinant DNA duplexes. For simplicity, only one set of possible recombinant duplexes is shown. The list of proteins involved in each step is not exhaustive.

In the absence of functional RecBCD other nucleases and helicases can provide the proper substrate for RecA binding [8]. Genetic analyses have implicated the *E. coli RecE, RecJ,* and *RecQ* genes in recombination. The RecE protein is a dsDNA-specific exonuclease that preferentially degrades the strand with the 5' end [13]. RecJ is a ssDNA-specific 5' to 3' exonuclease [14] and RecQ is a DNA helicase [15].

Besides processed DSBs, another source of ssDNA in the cell are ssDNA gaps that are generated when a replication fork stalls and/or disassembles when it encounters a DNA lesion [16, 17]. Repair of ssDNA gaps not only requires RecA protein but is also dependent on the RecF, RecO, and RecR proteins. Although previous genetic studies suggested that RecF, RecO and RecR act at the same stage of recombinational processes, perhaps as a RecFOR complex [18], recent *in vitro* studies reveal distinct roles of RecFR and RecOR complexes in repair of ssDNA gaps. One of the functions of the RecF, RecO, and RecR proteins, together with the single-strand DNA binding protein (SSB), is to modulate the assembly of a RecA filament onto the ssDNA of the gap (see Figure 3). Formation of RecA-ssDNA filaments is greatly facilitated by SSB which removes DNA secondary structures to ensure contiguity of the filament [19]. Although SSB appears to be important for optimal activity of RecA in homologous recombination, there is a conflict between RecA and SSB *in vitro*. Because the binding of RecA and SSB to ssDNA is mutually exclusive, RecA must be added prior to the addition of SSB [20]. The inhibition of Rec A filament nucleation by prebound SSB can be overcome by the

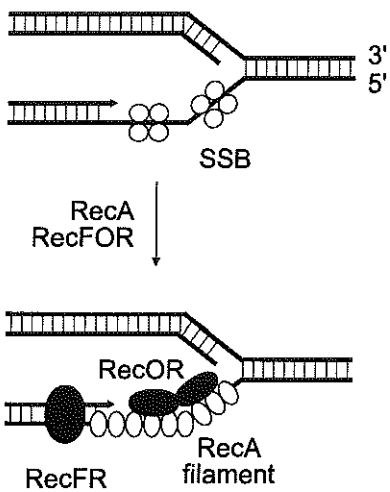

Figure 3. Role of homologous recombination in DNA replication; model for the function of RecF, RecO, and RecR in repair of ssDNA gaps, generated at stalled replication forks. The RecA filament can not assemble on ssDNA that is prebound with SSB. A complex of the RecOR proteins facilitates RecA assembly on the ssDNA of the gap and prevents the end-dependent disassembly of the RecA filament. Filament extension into the adjoining duplex DNA is halted by a complex of the RecFR proteins, bound to dsDNA near the gap. Interaction of the RecFR complex with the replication machinery might be required for replication restart after the DNA lesion is repaired. This figure is modified from Cox [16].

addition of the RecO and RecR proteins [21]. Furthermore, the RecOR complex prevents end-dependent disassembly of the RecA filament, which occurs at the filament end nearest the 5' end of the DNA [22]. In both cases, the RecO and RecR proteins act together, with no effects seen with either protein alone. During RecA-dependent post-replication repair, excessive extension of the RecA filament into the adjoining duplex DNA is halted by the presence of the RecF and RecR proteins [23]. In addition, the apparent interaction of the RecFR complex with the replication machinery might be required for replication restart after the DNA lesion is repaired or bypassed [24, 25]. The experiments on the *E. coli* RecF, RecO, and RecR proteins highlight the important role of homologous recombination in DNA replication. Under normal laboratory growth conditions of *E. coli*, 10-50% of the replication forks initiated at the replication origin are interrupted and require recombination functions for their origin-independent restart [16].

To produce mature recombinant molecules, the strand exchange intermediates made by RecA need to be processed by other enzymes, including DNA replication factors and the RuvA, RuvB, and RuvC proteins (Figure 2C, D, E) [26]. Catalysis of strand exchange by RecA eventually results in physical joining of the recombination partners through so-called Holliday junctions (Figure 2D). Movement of these junctions is known as branch migration and extends heteroduplex DNA. Biochemical studies showed that RuvA is a structure specific DNA-binding protein that has high affinity for Holliday junctions [27]. The RuvB protein is a DNA-dependent ATPase, which forms hexameric ring structures around DNA and promotes branch migration [28]. RuvA acts by targeting the hexameric RuvB structure specifically to the junction. The RuvC protein is an endonuclease that specifically binds to Holliday junctions and mediates their resolution [28]. *In vitro* reconstitution experiments showed that RecA, RuvA, RuvB, and RuvC can act simultaneously [29]. In this coupled reaction, the RuvAB proteins serve as an intermediary between the formation of Holliday junctions by RecA and their resolution by RuvC.

In addition to the limited number of key recombination proteins mentioned above, a large set of additional *E. coli* recombination proteins has been identified. A description of their activities and functions can be found in a number of reviews[9,30].

3 DSB REPAIR IN *SACCHAROMYCES CEREVISIAE*; THE *RAD52* PATHWAY

Extensive genetic analyses established that DSBs are efficiently repaired through homologous recombination in the budding yeast *S. cerevisiae* [31, 32]. Mutants in the so-called *RAD52* epistasis group are defective in DSB repair through homologous recombination and display sensitivity to ionizing radiation and the alkylating agent methyl methanesulfonate (MMS). *RAD52* group genes, isolated by

complementation of the mutant phenotype, include *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11,* and *XRS2.*

An important clue to the function of the *RAD52* group proteins came from the observation that ScRad51 showed sequence similarity to the *E. coli* RecA strand exchange protein [33]. Subsequent biochemical studies showed that the ScRad51 protein promotes ATP-dependent homologous pairing and DNA strand exchange [34]. As with the RecA protein, assembly of a presynaptic nucleoprotein filament composed of ssDNA coated with ScRad51 protein initiates homologous DNA pairing, which is followed by strand exchange (see Figure 2B). A ssDNA-binding protein known as RPA (replication protein A) stimulates filament formation by ScRad51, presumably by removing secondary structure from the ssDNA analogous to the function of SSB in RecA-mediated strand exchange [35]. Because of the shared ability of ScRad51 and RPA to bind ssDNA, displacement of RPA is necessary for the formation of an active presynaptic filament. Indeed, it has been shown that although RPA is required for ScRad51-promoted strand exchange, it inhibits exchange *in vitro* when incubated simultaneously with ScRad51 and ssDNA [36-38]. Since RPA is also present *in vivo* during ScRad51 nucleoprotein filament formation, mechanisms must exist to allow filament formation in the presence of RPA. Recent biochemical studies revealed that the inhibition of ScRad51-promoted strand exchange by RPA, is overcome when ScRad52 is incubated together with ScRad51, RPA and ssDNA, followed by the addition of homologous dsDNA [36-38]. While the role of ScRad52 as a mediator between ScRad51, RPA, and ssDNA has been established, the molecular mechanism of this mediation remains to be determined. Recently, a gene with sequence similarity to *ScRAD52*, named *ScRAD59*, has been identified [39], but the function of the encoded protein has not yet been identified.

In addition to ScRad52, the proteins ScRad55 and ScRad57 have been implicated in overcoming the inhibitory effect of RPA during nucleation of ScRad51 onto ssDNA [40]. ScRad55 and ScRad57 show limited amino acid sequence similarity to ScRad51. However, there is no evidence for filament formation by these proteins. Instead, they form a stable heterodimer. Inclusion of this heterodimer in reaction mixtures containing ScRad51 and RPA results in a marked stimulation of strand exchange [40].

ScRad54 is a member of the SNF2/SWI2 family of proteins that have been implicated in many aspects of DNA metabolism such as transcription, repair, and recombination [41]. The protein family is characterized by conserved sequence motifs found in DNA helicases [42]. However, none of the SNF2/SWI2 family proteins have been shown to possess helicase activity. It has been suggested that the conserved motifs that confer helicase activity to some proteins provide a more general function of which helicase activity represents a subset [43]. Recent biochemical experiments revealed a potential function of ScRad54. Although ScRad51 promotes pairing between ssDNA and its homologous dsDNA, the reaction is inefficient in comparison to that catalyzed by *E.coli* RecA [2]. The first heteroduplex DNA intermediate generated during recombination is a D-loop structure between linear

ssDNA and supercoiled DNA (see Figure 2B). ScRad51 protein alone is incapable of forming such a D-loop. ScRad54 significantly increases the pairing rate and stabilizes D-loop formation [44]. The conserved amino acid motifs of ScRad54 are also found in the *E. coli* DnaB and RuvB proteins [45]. A common feature of these molecular motor proteins is their ability to translocate along DNA. If ScRad54 contains a similar activity, ScRad54 could function as a processivity factor for ScRad51-mediated strand exchange. The recently identified interaction between ScRad54 and ScRad51 [46, 47], is consistent with such a function. ScRad54-mediated processivity of strand exchange might be especially important in the context of chromatin or to overcome the inhibitory effect of heterologous DNA sequences that can occur when the DSB is repaired from the homologous chromosome instead of the sister chromatid.

The many genetic and physical interactions detected among ScRad51, ScRad52, ScRad54, ScRad55, ScRad57, and RPA suggest that the proteins of the *RAD52* DSB repair pathway function in the context of a highly coordinated and ordered protein machine. The elucidation of molecular mechanisms by which these proteins act and the order in which they perform their function provide a formidable challenge for the near future.

Although the *RAD50, MRE11*, and *XRS2* genes clearly function in the *RAD52* pathway during meiotic recombination [31], their role during homologous recombination in mitotic cells is less clear. Recently, these genes have also been implicated in DSB repair through DNA end-joining [5]. The ScRad50, ScMre11 and Xrs2 proteins form a complex [48], which is consistent with the similar phenotype of the respective mutants. The discussion of their function is postponed to Section 6.

4 DSB REPAIR IN MAMMALIAN CELLS; THE DNA END-JOINING PATHWAY

In a manner analogous to the isolation of genes involved in DSB repair in *S. cerevisiae*, a collection of ionizing radiation sensitive Chinese hamster ovary cell lines proved critical in identifying mammalian DSB repair genes. However, in contrast to the homologous recombination genes identified in *S. cerevisiae*, the isolated mammalian genes were found to be involved in DSB repair through DNA end-joining (see Figure 4) [6]. Interestingly, it turned out that a number of these mutants were not only defective in processing the ionizing radiation induced DSBs, but also in processing the naturally occurring DSBs during V(D)J recombination which is required for the generation of immunoglobulins and T cell receptors.

The realization that V(D)J recombination and repair of ionizing radiation induced DNA damage require some of the same proteins resulted from the observation that *scid* mice and their derived cells were ionizing radiation sensitive [49, 50]. Originally, *scid* mice had been identified as having severe combined immune deficiency which was later shown to be due to defective V(D)J recombination [51]. The gene defective in *scid* cells was shown to encode the catalytic subunit of DNA-

dependent protein kinase (DNA-PKcs) [52-54]. The additional component of DNA-PK is a heterodimer of the Ku70 and Ku80 proteins [55]. Like *scid* mice and cells, *Ku70* and *Ku80* knockout mice and cells display ionizing radiation sensitivity and are defective in V(D)J recombination [56-58].

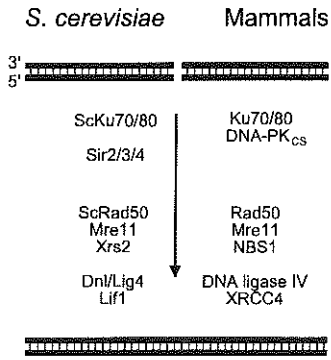

Figure 4. DSB repair through DNA end-joining. Proteins implicated in DNA end-joining are indicated. DNA end-joining probably involves interaction of the Ku70/Ku80 heterodimer with the ends, resulting in the targeting of DNA-PKcs to the damaged site and processing of the ends by the exonuclease activity of Mre11. The order of entry and exit of proteins during the DNA end-joining process is still unclear. See text for details.

While studies on the mutant rodent cells and mice established the involvement of the DNA-PK subunits in DNA end-joining, biochemical analyses provided additional insight into their role in DNA end-joining. The Ku70/80 heterodimer was shown to bind with high affinity to DNA ends [59]. This property is consistent with a role for the Ku heterodimer in damage recognition during the first steps of DSB repair. While bound to the ends, Ku could be involved in a number of processes. First, it could serve to protect the ends from nucleolytic degradation [60, 61]. Second, since Ku can transfer directly between DNA molecules with complementary overhangs, the heterodimer possibly functions in juxtaposing broken DNA ends [62]. Third, Ku might have a role in attracting other proteins to the break. Upon binding to a DNA end, Ku can attract DNA-PKcs and stimulate its kinase activity [63]. *In vitro*, DNA-PK phosphorylates several transcription factors and other DNA-binding proteins but the functional significance of the phosphorylations are still unclear [64]. Interestingly, *in vitro* DNA-PK undergoes autophosphorylation of all three subunits, which correlates with inactivation of the serine/threonine kinase activity of DNA-PK, probably caused by reduced affinity of phosphorylated DNA-PKcs for the Ku heterodimer [65]. Inactivation of the kinase activity of DNA-PK might be necessary to abrogate its potential DNA damage signaling functions.

After DSB recognition, processing of the damage needs to occur. Proteins that are likely to have a role in processing the DSB during DNA end-joining are human Rad50 and Mre11 (see Figure 4). A role for the *S. cerevisiae* homologs of these proteins in DNA end-joining has clearly been demonstrated and is discussed in Section 6. Immunoprecipitation experiments have revealed that hRad50 and hMre11 are part of the same multiprotein complex [66]. Biochemical experiments demonstrated that hMre11 has 3' to 5' exonuclease activity that is increased when

hMre11 is in a complex with hRad50 [67]. Immunofluorescence experiments have shown that upon induction of DSBs in mammalian cells, hRad50 and hMre11 redistribute to form nuclear foci [68]. In an inventive experiment, where part of the nucleus was shielded from ionizing radiation, it was found that hRad50-hMre11 foci are formed only in the irradiated volume [69]. Together, these experiments support a direct involvement of the hRad50/hMre11-containing complex in nucleolytic processing of DNA ends. Besides a role in DNA end-joining, the complex is likely to have additional, yet unidentified, functions. Disruption of *MRE11* in mice or cells results in a lethal phenotype [70]. In contrast, disruption of components of DNA-PK results in viable animals showing the *scid* phenotype and hypersensitivity to ionizing radiation [49, 56-58, 71].

One of the final steps in sealing the DSB is ligation of the strands. Of the four mammalian DNA ligases [72], DNA ligase IV has been implicated in the DNA end-joining process because of its association with the XRCC4 protein. Mammalian cells with mutations in *XRCC4* are sensitive to ionizing radiation and are defective in formation of signal and coding joints during V(D)J recombination [6]. The *XRCC4* gene has been cloned but the sequence of the encoded protein did not reveal a possible function [73]. Recently, it became clear that XRCC4 forms a protein complex with DNA ligase IV [74, 75]. In addition, in *S. cerevisiae* the DNA ligase IV homolog Dnl4/Lig4 is proposed to function in the ligation step during DNA end-joining (see Section 6).

5 THE *RAD52* PATHWAY IN MAMMALIAN CELLS

As none of the screens for ionizing radiation sensitive hamster cell lines resulted in mutants with a defect in homologous recombination genes, mammalian cells appeared to depend mainly on DNA end-joining for ionizing radiation resistance. This was supported by the fact that extensive biochemical experiments failed to identify eukaryotic strand exchange proteins with the hallmarks of RecA; ATP binding and hydrolysis, and nucleoprotein filament formation [8]. The isolation of the mammalian genes with sequence similarity to *ScRAD51* and *RecA* [76] was the beginning of a revitalized effort to elucidate the mechanism and the role of homologous recombination in mammals. The search for open reading frames similar to human *RAD51 (hRAD51)* revealed the existence of three additional genes with sequence similarity to *hRAD51*, named *hRAD51B, hRAD51C* and *hRAD51D* [77-79], as well as the meiosis-specific homolog *HsDmc1* [80]. Based on their amino acid sequence the human XRCC2 and XRCC3 proteins, which complement the ionizing radiation sensitivity of Chinese hamster cell lines irs1 and irs1SF, respectively, were recently identified as two additional members of the *RAD51*-related protein family [81]. In addition to these *RAD51*-related genes, mammalian genes with amino acid sequence identity to *ScRAD50, ScRAD51, ScRAD52, ScRAD54,* and *ScMRE11* have now been isolated [1, 4]. Amino acid sequence similarity between yeast and mammalian *RAD52* group gene products range from 50-83%. Given the large

evolutionary distance between fungi and humans this conservation implies an important function for the mammalian *RAD52* group proteins. For a number of the proteins evidence for functional conservation has been obtained.

Biochemical studies have shown that hRad51, like ScRad51, promotes ATP-dependent homologous pairing and DNA strand exchange [82-84]. In yeast and mammals, Rad51 and Rad52 have been shown to interact [85, 86]. During homologous recombination, the hRad51 protein coats the ssDNA to form a filament that scans the genome for a homologous dsDNA sequence. Homologous DNA pairing results in the formation of a joint molecule (Figure 2B). Efficient joint molecule formation occurs when ssDNA is incubated with saturating amounts of hRad51 and does not occur when hRad51 is present at subsaturating amounts. However, when ssDNA is incubated with hRad52, followed by the sequential addition of subsaturating amounts of hRad51, joint molecule formation proceeds efficiently [87]. Thus, in both yeast and mammals, Rad52 is required for the critical early steps of genetic recombination.

Although hRad51 and hRad52 seem to act in close collaboration in these first steps of homologous recombination, the effect of a mutation in either *RAD51* or *RAD52* in mice differs dramatically. Targeted disruption of murine *RAD51* (*mRAD51*) results in embryonic lethality [88, 89], while disruption of *mRAD52* results in viable mice with no evident phenotypes [90]. The embryonic lethality of *RAD51* knockout mice and cells suggests a role for Rad51 in cellular proliferation. Another indication for a role for Rad51 during proliferation came from experiments using chicken DT40 cells, in which the endogenous chicken *RAD51* loci were inactivated. The cells were kept alive by expression of hRad51 from a repressible promoter. Depletion of hRad51 from these cells, resulted in the accumulation of chromosomal breaks, followed by cell death [91]. A role for mammalian Rad51 during cell proliferation was also suggested by studies that showed that hRad51 concentrated into multiple discrete nuclear foci during S-phase [92]. In addition, the percentage of foci-containing nuclei increased upon treatment with ionizing radiation [93].

The effects of *RAD52* mutants in *S. cerevisiae* and mouse embryonic stem cells are also very different. Homologous integration in *S. cerevisiae* is virtually eliminated in mutant *rad52* strains [94], while it is only two-fold reduced in mouse *RAD52* knockout embryonic stem cells [90]. It is not clear, why the disruption of *RAD52* has such different effects in *S. cerevisiae* and mouse cells. A possible explanation could be that Rad52 functions in mammals are redundant. If ScRad59 homologs also exist in mammals then it is possible that this protein could perform some of the functions of Rad52.

Functional conservation was also shown for the mammalian homolog of *ScRAD54*. The *hRAD54* gene encodes a DNA-dependent ATPase that can partially complement the MMS sensitivity of a *rad54Δ S. cerevisiae* strain [95, 96]. In addition, disruption of *RAD54* in mouse embryonic stem cells and chicken cells reduced ionizing radiation resistance and homologous recombination [97, 98]. These observations linked a decrease in homologous recombination to ionizing radiation

sensitivity. Interestingly, *RAD54* knockout mouse embryonic stem cells are also sensitive to the crosslinking agent mitomycin C [97]. In contrast, DNA end-joining defective cells, such as those derived from *scid* mice, are not mitomycin C sensitive [49]. This observation provides an explanation for the existence of multiple DSB repair pathways; although their functions can sometimes overlap, they can also have specialized functions.

5.1 Possible functions of mammalian Rad54

The main characteristic features of the mammalian Rad54 protein as described in this thesis, point to several potential functions of mammalian Rad54. Based on its amino acid sequence, Rad54 belongs to the superfamily of DNA-dependent ATPases [42]. It has been suggested that the conserved motifs that confer helicase activity to some members of this family provide a more general function of which helicase activity can be a subset [43]. This more general activity could be the ability to track along DNA at the expense of ATP hydrolysis [99]. This potential translocation of Rad54 along DNA could be useful in at least three different stages of homologous recombination that are discussed below.

(1) The translocation of Rad54 might provide processivity to Rad51-mediated strand exchange. *In vitro*, joint molecule formation by the *E. coli* RecA protein is followed by an extensive strand exchange reaction that requires the hydrolysis of ATP, while the extent of strand exchange by hRad51 is limited [2, 8]. hRad51 hydrolyzes ATP at a rate that is approximately 200-fold lower than that observed for RecA [2]. This strongly reduced rate of ATP hydrolysis could result in the limited strand transferring ability of hRad51 compared to RecA. Although it can not be ruled out that the *in vitro* system does not accurately reflect the *in vivo* activities of hRad51, the *in vivo* function of hRad51 might indeed be limited to the formation of these initial joints, which could then be extended by other enzymes. A possible function of hRad54 in stabilization or extension of the hRad51-mediated joints is substantiated by three different observations described in Chapter 7. First, DNA damage can induce an interaction between mRad54 and mRad51; second, hRad54 mediates dsDNA unwinding; third, mRad54 is required for DNA damage induced mRad51 foci formation.

While translocating along the DNA and simultaneously interacting with Rad51, Rad54 could provide the necessary reaction conditions for the strand exchange reaction. The creation of these proper reaction conditions might require the ATP-dependent DNA unwinding activity of Rad54. Both hRad54 and ScRad54 belong to the SNF2/SWI2 subfamily of the helicase superfamily of proteins. They are dsDNA-dependent ATPases without detectable helicase activity [44, 96]. Recently, it has been shown that the human SNF2/SWI2-containing protein complex can remodel nucleosome structure, suggesting that human SNF2/SWI2 acts by facilitating an exchange between normal and altered, more accessible, nucleosome conformations [100]. Rad54 might have an analogous function. Rad54-mediated

DNA unwinding might result in displacement of histones that could be inhibitory to homologous pairing and thereby facilitates the formation of a more extensive Rad51-ssDNA filament. This potential effect of Rad54 on nucleoprotein filament formation would explain why Rad54 is required for DNA damage-induced Rad51 foci formation, since a more extensive filament will result in a higher local concentration of Rad51 protein that can then be detected by immunofluorescence.

Besides at the chromatin level, Rad51-mediated DNA unwinding could help stabilize or extend Rad51-promoted joint-molecules more directly. Because the ssDNA in the Rad51-ssDNA filaments is in an extended conformation [2, 8], unwinding of the dsDNA by Rad54 will facilitate the pairing between the homologous ss- and dsDNA. Indeed, ScRad54 has been shown to increase the pairing rate of homologous ss- and dsDNA, possibly through the stabilization of D-loop formation [44].

Recently, a second protein has been identified that is required for DNA damage-induced Rad51 foci formation. Similar to the observation that genotoxic stress failed to induce mRad51 foci in *mRAD54*$^{-/-}$ cells (Chapter 7), DNA damage did not induce Rad51 foci in the Chinese hamster ovary cell line irs1SF, which is defective in *XRCC3* [101]. The protein has amino acid sequence similarity to hRad51 and interacts with hRad51 [81]. Although both Rad54 and XRCC3 promote the assembly or stabilize a multimeric form of Rad51, they probably function by different mechanisms. As described above, the critical action of Rad54 on the Rad51-mediated reaction is most likely through its effect on the dsDNA recombination partner. In contrast, given its amino acid sequence XRCC3 probably functions, at least in part, by affecting the Rad51 nucleoprotein filament. In *S. cerevisiae*, the ScRad51 homologs ScRad55 and ScRad57 can form a heterodimer that can stimulate ScRad51-mediated strand exchange at substoichiometric amounts relative to ScRad51 [40]. There is no evidence for ScRad55 or ScRad57 nucleoprotein filament formation. Instead, the two proteins form a stable heterodimer. Through the interaction of ScRad55 with ScRad51, this heterodimer might function by increasing the cooperativity of ScRad51 binding to ssDNA. In a manner analogous to ScRad55, XRCC3 could help mammalian Rad51 in DNA strand exchange.

(2) A second stage in homologous recombination where translocation of hRad54 could exert its function is in the process of branch migration. Late recombination intermediates consist of two duplex molecules linked by a Holliday junction. The Holliday junction, or branched structure, can migrate which results in the formation of heteroduplex DNA between the two recombining partner DNAs. In *E. coli*, the RuvB DNA-dependent ATPase promotes branch migration of Holliday junctions to extend regions of heteroduplex DNA. RuvB acts together with RuvA, which provides DNA-binding specificity by targeting RuvB to the Holliday junction. In addition, RuvA activates the helicase activity of RuvB, which is an essential activity for the promotion of branch migration [99]. The idea is that the helicase activity of RuvB drives branch migration by disrupting intra-partner base pairing which is followed by formation of inter-partner base pairing. Currently

there are no indications that enzymes involved in the process of branch migration, such as RuvA and RuvB, are conserved from *E. coli* to mammals. However, given the effect of Rad54 on homologous recombination and its shared amino acid sequence motifs with RuvB, it could function analogously to RuvB in promoting branch migration. If Rad54 functions in branch migration, then this would predict the existence of a helicase-activating and/or Holliday junction-targeting RuvA-like activity in eukaryotes, since no helicase activity of Rad54 by itself has been detected [44, 96].

(3) A third possible function requiring Rad54 translocation along DNA during homologous recombination could be its involvement in the turnover of the hRad51-DNA filament through disruption of hRad51-DNA binding. This would be analogous to the function of the *S. cerevisiae* Mot1 protein, which is also a SNF2/SWI2 family member. Mot1 action leads to the dissociation of the essential transcription factor TATA box-binding protein (TBP) from DNA. Furthermore, experiments with hybrid proteins between Mot1 and other SNF2/SWI2 family members indicate that other members of this family also function by mediating the ATP-dependent dissociation of protein-DNA complexes [102]. If Rad54 functions analogously to Mot1, then Rad54-mediated removal of Rad51 from joint molecules could stimulate homologous recombination in two ways. First, it could allow access of other proteins to the joint molecule that are required for its further processing, such as DNA polymerases. Second, Rad51 removal could prevent reversal of the Rad51-dependent strand exchange reaction. This role for hRad54 in the turnover of the hRad51-DNA filament would predict that the initial formation of a multimeric form of Rad51 is still possible in *mRAD54*$^{-/-}$ cells. However, in the light of our recent finding that DNA damage fails to induce mRad51 foci in *mRAD54*$^{-/-}$ cells (Chapter 7), a Mot1-like function for hRad54 is unlikely. The availability of purified hRad54 protein and the *in vitro* formation of joint molecules by hRad51 and hRad52 provide useful tools to discriminate between the different hRad54 functions suggested above.

6 THE DNA END-JOINING PATHWAY IN *S. CEREVISIAE*

Although the genetic screens for ionizing radiation sensitive yeast mutants had been very successful in identifying the role of homologous recombination in DSB repair, they failed to identify a role for DNA end-joining. Instead, biochemical analysis led to the discovery of a protein complex with DNA end-binding activities that were similar to the mammalian Ku complex [103, 104]. In addition, sequencing of the *S. cerevisiae* genome revealed genes encoding proteins with ~20% identity to Ku70 and Ku80 [103, 104]. Like the mammalian Ku proteins, ScKu70 and ScKu80 form a stable complex that has DNA end-binding activity. To prove the physiological significance of the Ku proteins in yeast, a crucial step was to knockout the *RAD52* DSB repair pathway. Only when homologous recombination was abolished by

deletion of *ScRAD52*, or the deletion of the homologous template, the subsequent deletion of *ScKu70* led to an increased sensitivity to ionizing radiation [105, 106]. This clearly illustrates why previous genetic screens for radiosensitive yeast mutants did not identify components of the yeast Ku-dependent repair systems.

Recently, *ScRAD50, ScMRE11,* and *XRS2* have been implicated in the DNA end-joining pathway. Originally, these genes were found to be part of the *RAD52* pathway. This classification was mostly based on their role in meiotic recombination. During meiosis the genes are required for the introduction and processing of meiosis specific DSBs [48, 107]. Their role in mitotic homologous recombination is less clear. Depending on the type of homologous recombination that is analyzed, an increase, no effect or a decrease in homologous recombination is seen in the absence of these genes [108]. Evidence for the involvement of ScRad50 in DNA end-joining came from experiments that identified *ScRAD50* as the first gene required for random integration of transfected DNA in yeast [94]. Subsequently, the requirement for ScRad50 in the joining of DNA ends, created by HO endonuclease, was revealed [109]. The role of the ScRad50/ScMre11/Xrs2 complex in meiotic recombination and mitotic DNA end-joining, but not (necessarily) in mitotic homologous recombination, points to a role in DNA end processing in meiotic recombination rather than a role in the subsequent homology-dependent repair step. The role of the ScRad50/ScMre11/Xrs2 complex in DNA end processing could be an enzymatic and a structural one. Its enzymatic role could be mediated through ScMre11. This protein has limited but significant sequence homology with the SbcC subunit of the *E. coli* ATP-dependent nuclease SbcCD [110]. In addition, the hMre11 protein is an exonuclease [67]. Nuclease activity of the ScRad50/ScMre11/Xrs2 complex could be involved in processing the DSB for subsequent repair. Such a role could be essential for the repair of ionizing radiation induced DSBs, which most likely possess damaged termini that preclude repair by simple ligation. Evidence for a structural role of the protein complex comes from studies using a plasmid recircularization assay. Introduction of linearized plasmids with cohesive ends in yeast cells results mostly in recircularized plasmids, that have retained the unique restriction site, used for linearization, without any evidence of degradation of the DNA ends [103]. The frequency of recircularization in the *ScRAD50* mutant is reduced. Since no nuclease activity is required in this assay, the result implies that ScRad50 complex has an active role in rejoining of DNA ends. A structural role of ScRad50 is consistent with the presence of amino acid sequence motifs in the protein, that are also found in SMC group proteins that are involved in chromosome condensation and segregation [111].

Although the recircularization assay provides a convenient method for the analysis of DSB repair, the challenge is to explain how a chromosomal DSB is repaired in the context of chromatin. Recently, it was found that three yeast proteins, associated with transcriptional silencing of telomeric DNA, are also involved in DSB repair by DNA end-joining [112-114]. In telomere silencing, the silencing factors Sir2, Sir3 and Sir4 bind DNA via the Rap1 protein, which binds to

specific sequences of telomeric regions [115]. Using the yeast two-hybrid system, it was discovered that Sir4 interacts with ScKu70. As ScKu70 can bind to DNA ends [104], it might help to recruit Sir4 to the DNA ends of the DSB. Sir4, in turn, could recruit Sir2 and Sir3. This is supported by the finding that disruption of the *SIR2, SIR3* or *SIR4* genes results in increased ionizing radiation sensitivity and defects in DNA end-joining in a *RAD52* knockout background [112, 113]. In contrast to DSB repair by homologous recombination, for which chromatin structure may complicate the search for homology, repair by DNA end-joining could take advantage of chromatin structure to keep the two DNA ends of a DSB in close proximity.

Joining of DNA strands is an essential final step in DNA metabolism. DNA ligases, which catalyze these reactions, join together DNA strands during replication, repair, and recombination. While mammalian cells have four DNA ligases, termed ligase I-IV [72], the complete DNA sequence of the *S. cerevisiae* genome revealed only two DNA ligases; a DNA ligase I homolog, encoded by *CDC9*, and a DNA ligase IV homolog, encoded by *DNL4/LIG4* [116-119]. Both mammalian DNA ligase I and its yeast homolog Cdc9 are essential replication factors that mediate joining of Okazaki fragments during lagging strand synthesis [120, 121]. Unlike *CDC9, DNL4/LIG4* is not essential for DNA replication. Cdc9 could not complement the function of Dnl/Lig4, which indicates that the ligases function in distinct and separate processes that require DNA ligation. The frequency of recircularization of linearized plasmids with cohesive ends is reduced by the mutation of *DNL4/LIG4*, suggesting that the gene encodes a crucial component of the DNA end-joining machinery [117-119]. A role for *DNL4/LIG4* in DNA end-joining is consistent with the observation that the frequency of plasmid recircularization is reduced to the same extent in *DNL4/LIG4* and *ScKu70* single mutants compared to the double mutant. However, unlike *ScKu70*, *DNL4/LIG4* does not function in telomeric length maintenance [119]. The role of *DNL/LIG4* in repair of ionizing radiation induced DNA damage is still controversial. An increase in sensitivity to ionizing radiation has been reported, but only when homologous recombination had been abrogated by deletion of *RAD52* [117, 119]. However, others found no further sensitization by deletion of *DNL/LIG4* in a *RAD52* knockout background [118]. A possible explanation for this discrepancy could be a difference in cell stage at the time of irradiation or a difference in the doses used. However, the results from the plasmid recircularization assay suggest that Dnl/Lig4 has an important role in the repair of DSBs through the DNA end-joining pathway. Recently, the functional analogy between mammalian DNA ligase IV and *S. cerevisiae* Dnl/Lig4 has been strengthened by the identification of Lif1. Lif1 (ligase 4 interacting factor 1) shows a moderate amino acid sequence similarity to XRCC4. Disruption of *LIF1* results in significantly increased sensitivity to ionizing radiation or MMS and defects in the plasmid recircularization assay when homologous recombination is inoperative [122].

A component of the mammalian DNA end-joining pathway that has not been unequivocally identified in the complete sequence of the *S. cerevisiae* genome is DNA-PKcs. It is not yet clear whether the absence of this protein reflects a

difference in DNA end-joining mechanism in yeast compared to mammalian cells, or whether its function is taken over by a different protein. However, the existence in *S. cerevisiae* of kinases with a low amino acid sequence identity to DNA-PKcs, such as Tel1, and the involvement of such factors in DNA damage signaling suggests that they might function together with the ScKu proteins in DNA end-joining in yeast [123]. Yeast cells defective in *TEL1* lose the majority of their $C_{1\text{-}3}A$ repeats at the end of their chromosomes [124]. A similar telomere-shortening phenotype is observed in strains lacking *ScKu70* or *ScKu80* [125, 126], implying that the Ku proteins and Tel1 function in the same telomere maintenance pathway. However, analysis of the efficiency of DNA end-joining after transfection of linearized plasmids in *TEL1* mutants, revealed wild-type levels of plasmid rejoining. Analysis of the rejoined products revealed that virtually all had been repaired by direct DNA end-ligation and had not suffered nucleotide loss or addition [113]. These results show that Tel1 is not an essential component of the Ku-dependent DNA end-joining pathway in yeast, at least for extra-chromosome substrates.

7 A SPECIALIZED DSB REPAIR PATHWAY; SINGLE-STRAND ANNEALING

Most of the recombination reactions in *S. cerevisiae* are carried out by the scRad51-mediated homologous DNA pairing reaction through the *RAD52* pathway. However, analysis of substrates containing a site-specific endonuclease cleavage site located within or between two directly repeated sequences established the presence of an alternative DSB repair pathway in yeast [127]. This pathway, called single-strand annealing, relies on the presence of two homologous sequences in direct orientation flanking the site of a DSB (Figure 5). According to the current models of single-strand annealing, bidirectional 5' to 3' degradation of the DSB ends leads to the exposure of complementary sequences, which then anneal. Subsequent removal of non-homologous 3' ended tails followed by DNA repair synthesis and ligation completes the process. Single-strand annealing results in deletion of one repeat along with the intervening sequence [128]. Analyses of DNA substrates injected into *Xenopus laevis* oocytes provided the first evidence for the existence of a similar single-strand annealing pathway in higher eukaryotes [129, 130]. Single-strand annealing in *S. cerevisiae* requires the *RAD1*, *RAD10*, *RAD52*, *MSH2*, *MSH3*, and *SRS2* gene products which are normally needed for three distinct DNA repair mechanisms.

The role of Rad52 in homologous recombination has been described in Section 3. Deletion of the *RAD52* gene results not only in reduced homologous recombination but also in significantly reduced DSB repair through single-strand annealing [128, 131]. The function of Rad52 in single-strand annealing can be explained by the finding that Rad52 promotes annealing of complementary sequences [132]. Although there is no genetic evidence in mammals for a role of mammalian Rad52

in single-strand annealing, a similar activity of hRad52 in promoting annealing of complementary DNA strands has been reported [133].

The role of Rad1 and Rad10, present in the cell as a heterodimeric endonuclease complex, in nucleotide excision repair has been well established [134]. Its nuclease activity is essential for the incision 5' of the DNA lesion. With the use of model repair and recombination intermediates, it was shown that cleavage by the Rad1-Rad10 endonuclease can occur at duplex-ss junctions. Moreover, cleavage occurs only on the strand containing the 3' ss tail [135]. Therefore, during single-strand annealing, the Rad1-Rad10 complex most likely removes the 3' non-homologous arms from the annealed duplex DNA. Analogous to the complex formation and activity of the Rad1 and Rad10 proteins in yeast, the homologous proteins in mammals, XPF and ERCC1, form a stable complex that can act as a structure-specific endonuclease [136]. Analysis of the DNA structural elements required for cleavage demonstrated that the XPF-ERCC1 complex, like the Rad1-Rad10 complex, efficiently removes 3' non-homologous arms at duplex-ss junctions [137]. Therefore, it is possible that XPF-ERCC1 also functions in single-strand annealing in mammals.

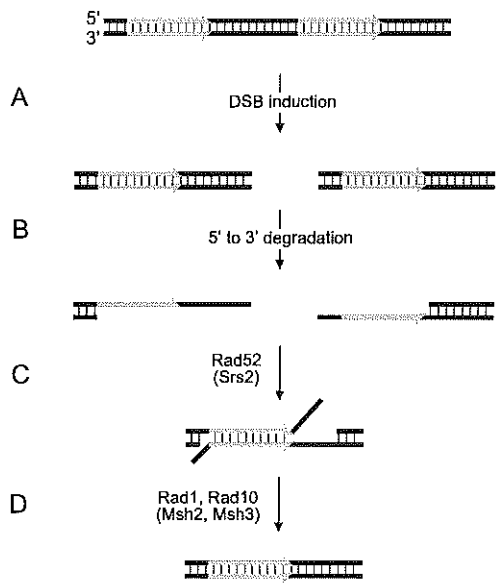

Figure 5. A model for DSB repair through single-strand annealing. **A**. Single-strand annealing can repair a DSB that occurs between or within repeated DNA sequences (indicated by gray arrows). **B**. Nucleolytic degradation of the DSB at the exposed 5'-ends will generate 3'-tailed single-strand regions. **C**. Annealing of complementary sequences can occur once the degradation has proceeded into the repeated sequences on both sides, probably with the aid of Rad52 and Srs2. **D**. The annealed intermediate is processed by Rad1-Rad10 mediated removal of the 3'-tails and sealing of the nicks resulting in a deletion. Msh2-Msh3 probably play a role in removing non-homology from the ends of recombining DNA.

Evidence that mismatch repair proteins might also be involved in the removal of 3'-ended ssDNA tails came from genetic experiments that indicated a requirement for *MSH2* and *MSH3* in spontaneous recombination events that also depend on *RAD1* and *RAD10* [138]. During mismatch repair, the Msh2-Msh3 complex recognizes mismatches or small heterologous loops in continuous duplex DNA

strands [139]. As Msh2-Msh3 can also recognize an entirely different substrate, such as a branched DNA structure with a free 3' end [140], it was suggested that Msh2-Msh3 facilitate removal of non-homologous DNA ends by Rad1-Rad10. Stabilization of the annealed intermediate by Msh2 and Msh3 was found to be particularly important when the length of the annealed sequence is short.

Recently, the 5' to 3' helicase Srs2 has also been implicated in single-strand annealing. It was suggested that Srs2 could unwind the two complementary DNA strands and thus increase the extent of pairing between the two strands in the single-strand annealing reaction [141]. This would, however, lead to the generation of larger deletions around the DSB.

The role of single-strand annealing in DSB repair is limited to special situations, because DSB repair through this pathway can only take place between directly repeated sequences. Moreover, DSB repair through single-strand annealing results in mutations. Therefore, DSB repair through DNA end-joining or homologous recombination is more general applicable.

8 CELL CYCLE

Another important cellular process that contributes to successful DSB repair is the activation of cell cycle checkpoints upon induction of DNA damage. Checkpoints are of crucial importance for the maintenance of genomic integrity of actively dividing cells, since they cause cells to pause and allow sufficient time to repair DNA damage before segregation of the replicated genome in the daughter cells. Mutations in genes involved in cell cycle arrest can lead to ionizing radiation sensitivity, such as found in Ataxia-telangiectasia (AT) and Nijmegen Breakage Syndrome (NBS) patients. The cellular phenotypes of AT and NBS are indistinguishable and include chromosomal instability, ionizing radiation sensitivity, and defects in cell cycle arrest upon irradiation [142]. A direct physical link between proteins involved in DSB repair and cell cycle regulation was recently demonstrated, with the identification of the gene, named *NBS1*, mutated in NBS patients [143-145]. By direct protein sequencing it was found that one of the unidentified components of the DSB repair complex hRad50/hMre11 was the NBS1 protein [145]. The *NBS1* gene only showed limited homology to the *S. cerevisiae XRS2* gene, the third component of the Rad50/Mre11 complex in yeast. The identification of the NBS1 protein as part of a multiprotein complex containing the hRad50 and hMre11 DSB repair proteins will help to integrate DSB repair in the intricate network of genes required for cell cycle regulation.

9 CONCLUSIONS AND FUTURE PERSPECTIVE

The importance of DSB repair for the maintenance of genomic integrity and the extensive use of ionizing radiation in anti-tumor therapy make it paramount to gain

more insight into the molecular mechanisms of DSB repair. Genetic screens for ionizing radiation sensitive yeast cells led to the identification of genes involved in DSB repair through homologous recombination. In contrast, similar screens for ionizing radiation sensitive mammalian cell lines, resulted in the identification of genes involved in DNA end-joining. This led to the notion that DSB repair occurs via different mechanisms in yeast and in mammalian cells. However, with the identification of additional DSB repair genes in the last few years, it has become clear that the two major DSB repair pathways are conserved from yeast to mammals. The next challenge is to clarify the molecular mechanisms for DSB repair. The biochemical activities of the individual gene products are rapidly being unraveled, which may facilitate the *in vitro* reconstitution of both the homologous recombination and DNA end-joining reactions.

There are various explanations for the fact that multiple pathways have evolved to coordinate the repair of DSBs. Homologous recombination and DNA end-joining potentially differ in fidelity of DSB repair. DSB repair through DNA end-joining can result in errors, as nucleotides at the break can be added or lost, resulting in incorrect joining. This argument is underscored by the fact that during V(D)J recombination the DNA end-joining machinery is used to create diversity in antibodies and T cell receptors. In contrast, homologous recombination repairs DSBs accurately by using the undamaged sister chromatid or homologous chromosome as a template to restore the lost information. Accurate repair is essential for a unicellular organism such as *S. cerevisiae* as most of its genome contains coding information. For a similar reason, homologous recombination might be the preferred mechanism of DSB repair in mammalian germline cells. Somatic mammalian cells could rely more on simple DNA end-joining for DSB repair, as a large fraction of their genome is no longer functional. Furthermore, the homologous recombination and DNA end-joining machinery might differ their relative importance during subsequent stages of the cell cycle and may show tissue, and damage specificity. Thus, although both DNA end-joining and homologous recombination contribute to the repair of DSBs in mammalian cells, specialized functions for either pathway may exist. An important challenge ahead is the evaluation of the relative contribution of the different DSB repair pathways to genomic stability and ionizing radiation resistance in different mammalian tissues and tumors. Given potential differences in fidelity of repair through the different pathways, this information is critical in assessing the risks due to ionizing radiation exposure and the differential sensitivity of tumor and normal cells.

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CHAPTER 2

Human and mouse homologs of the Saccharomyces cerevisiae RAD54 DNA repair gene: evidence for functional conservation

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Human and mouse homologs of the *Saccharomyces cerevisiae RAD54* DNA repair gene: evidence for functional conservation

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Background: Homologous recombination is of eminent importance both in germ cells, to generate genetic diversity during meiosis, and in somatic cells, to safeguard DNA from genotoxic damage. The genetically well-defined RAD52 pathway is required for these processes in the yeast *Saccharomyces cerevisiae*. Genes similar to those in the RAD52 group have been identified in mammals. It is not known whether this conservation of primary sequence extends to conservation of function.

Results: Here we report the isolation of cDNAs encoding a human and a mouse homolog of *RAD54*. The human (hHR54) and mouse (mHR54) proteins were 48 % identical to Rad54 and belonged to the SNF2/SWI2 family, which is characterized by amino-acid motifs found in DNA-dependent ATPases. The *hHR54* gene was mapped to chromosome 1p32, and the hHR54 protein was located in the nucleus. We found that the levels of *hHR54* mRNA increased in late G1 phase, as has been found for *RAD54* mRNA. The level of *mHR54* mRNA was elevated in organs of germ cell and lymphoid development and increased *mHR54* expression correlated with the meiotic phase of spermatogenesis. The *hHR54* cDNA could partially complement the methyl methanesulfonate-sensitive phenotype of *S. cerevisiae rad54Δ* cells.

Conclusions: The tissue-specific expression of *mHR54* is consistent with a role for the gene in recombination. The complementation experiments show that the DNA repair function of Rad54 is conserved from yeast to humans. Our findings underscore the fundamental importance of DNA repair pathways: even though they are complex and involve multiple proteins, they seem to be functionally conserved throughout the eukaryotic kingdom.

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Background

Homology-dependent repair of double-strand DNA breaks involves a complex recombinational repair pathway that requires a large number of proteins [1]. Double-strand breaks are induced by ionizing radiation which is often used in cancer therapy, and by radiomimetic agents, including endogenously produced radicals. Accurate repair of double-strand breaks is of pivotal importance in preventing chromosomal aberrations, such as translocations, which are often involved in carcinogenesis. In addition to the important role of double-strand break repair in mammalian somatic cells, the process also plays a vital role in germ cells. During meiosis, double-strand breaks are critical for homologous recombination, which is required for proper chromosome segregation and results in the generation of genetic diversity [2]. The processing of double-strand breaks is, therefore, an important cellular process, and its relevance extends beyond the repair of exogenously induced DNA damage.

Saccharomyces cerevisiae RAD52 epistasis group mutants are sensitive to ionizing radiation and to the alkylating agent methyl methanesulfonate (MMS), implicating the RAD52 group genes in double-strand break repair [3]. Mutations in the *RAD51*, *RAD52*, and *RAD54* genes cause the most sensitive phenotypes. The *rad51*, *rad52*, and *rad54* mutants are also defective in mating-type switching, a specialized mitotic recombination event [4]. The *RAD51*, *RAD52*, and *RAD54* genes have been isolated and characterized to some extent. The Rad51 protein is the homolog of the *Escherichia coli* recombination protein RecA [5]. These proteins, which are 22 % identical in amino-acid sequence, form a nucleoprotein filament with single-stranded DNA and mediate homologous DNA pairing and strand exchange [6–9]. The role of Rad52 in homologous recombination and double-strand break repair is less well understood. Rad52 can interact, either directly or indirectly, with Rad51 [5,10]. On the basis of its amino-acid sequence, Rad54 belongs

to the still expanding superfamily of DNA-dependent ATPases. Some members in this superfamily have DNA helicase activity. The superfamily is divided into subfamilies; Rad54 is a member of the SNF2/SWI2 subfamily [11]. Proteins in the superfamily are characterized by seven common motifs, including a bipartite purine nucleotide-binding motif. Proteins from a variety of species are found in the SNF2/SWI2 family and its members have been implicated in transcriptional regulation, recombination and a number of DNA repair pathways [12].

Recently, it has become clear that the primary sequence of some genes in the RAD52 epistasis group is conserved throughout evolution. Genes with sequence similarity to *RAD51* and *RAD52* have been found in numerous species, including *Schizosaccharomyces pombe*, chicken, mouse and humans [13–20]. Recently, a *S. pombe RAD54* (*rhp54*) and a human homolog of *S. cerevisiae MRE11* have been isolated [21,22]; mutants in *S. cerevisiae MRE11* are defective in meiotic recombination and the gene has been placed in the RAD52 epistasis group [23]. It is not known whether the function of any of the mammalian RAD52 group genes is conserved. Here we report the isolation of cDNAs encoding a human and a mouse homolog of Rad54, named hHR54 and mHR54, respectively. We show that *RAD54* and *hHR54* are true homologs because hHR54 can functionally substitute for Rad54 in repair of MMS-induced DNA damage. It appears that DNA repair pathways are of fundamental importance because even though they are highly complex multiprotein pathways they seem to be conserved throughout evolution.

Results

Isolation of human and mouse cDNAs encoding homologs of *RAD54*

We isolated a human and a mouse cDNA each encoding a protein with extensive similarity to Rad54. Amino-acid sequence conservation between the SNF2/SWI2 family member ERCC6 from humans and Rad54 was exploited to design two degenerate oligonucleotide primers corresponding to stretches of conserved amino acids near motif VI (see Materials and methods). Using RT-PCR (reverse transcribed polymerase chain reaction), we isolated a cDNA fragment from chicken bursal mRNA that encoded 41 amino acids with 83 % identity to Rad54 and 68 % identity to ERCC6 [24]. The PCR product was used to isolate a 1.3 kb DNA fragment from a chicken testis cDNA library that encoded 415 amino acids with 56 % identity and 72 % similarity to Rad54 (amino acids 389 to 810). The 1.3 kb DNA fragment was used to screen human and mouse testis cDNA libraries. The sequence of overlapping cDNA clones that hybridized to the chicken-derived probe was determined. One of the human cDNAs contained a 2241 bp open reading frame predicted to encode a protein of 747 amino-acids with a calculated molecular weight of 84.3 kDa. The putative start codon was not in the context of an optimal Kozak translation initiation sequence, but it was preceded by stop codons in all three reading frames at positions –26, –18, and –10, respectively. The mouse cDNA also contained a 2241 bp open reading frame, encoding a protein that was 94 % identical to the human homolog.

Figure 1 shows the comparison of the predicted amino-acid sequences of the proteins encoded by the human and mouse cDNAs with Rad54 and rhp54. Because of the high amino-acid sequence identity between the mammalian proteins and Rad54, we refer to them as hHR54 (for human homolog of Rad54) and mHR54 (for mouse homolog of Rad54). The amino-acid sequence identities and similarities are quantitated in Table 1. The identity and similarity between Rad54, rhp54, hHR54, and mHR54 extended over their entire length. The overall identity between the mammalian and yeast proteins was 48 % or higher. The seven motifs characteristic of SNF2/SWI2 family members were all present in hHR54 and mHR54 (Fig. 1). These motifs, including a Walker-type nucleotide-binding motif, spanned approximately 460 amino acids. We refer to this region as the DNA-dependent ATPase domain. The identity among the four proteins was highest in this domain, but the amino and carboxyl termini also showed significant

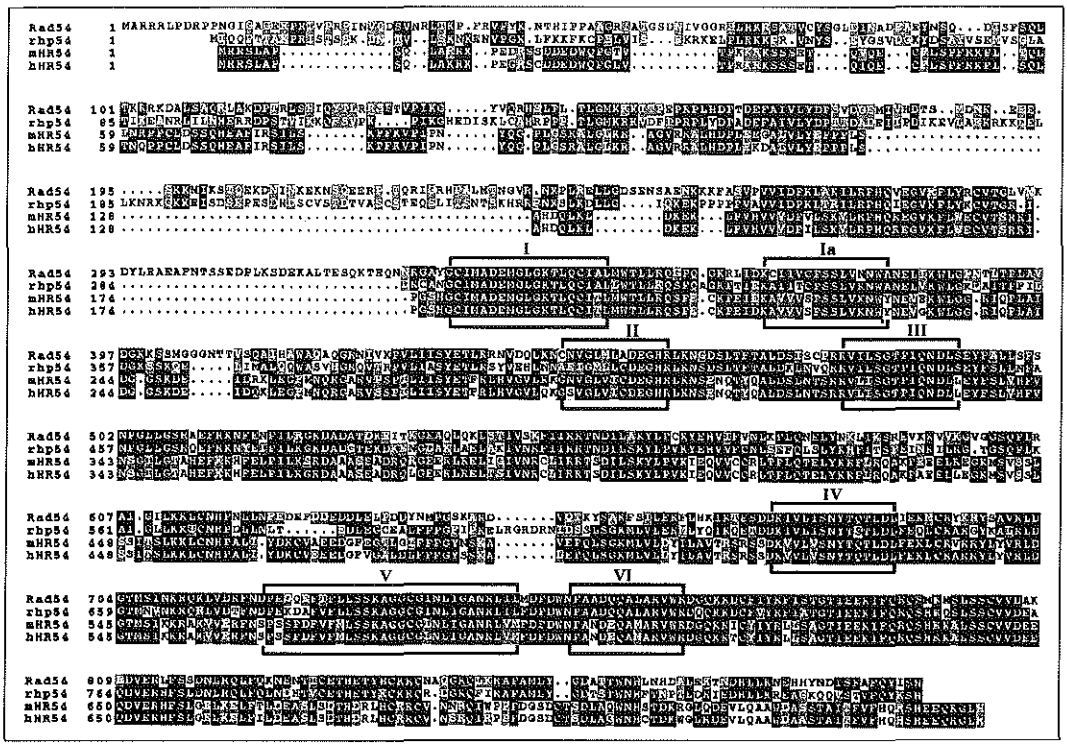
Table 1

Quantitation of amino-acid sequence identities and similarities among Rad54 homologs.

	S. pombe rhp54		hHR54	
	% Identity	% Similarity	% Identity	% Similarity
***S. cerevisiae* Rad54**				
Overall	58	74	48	68
Amino terminus	34	60	32	55
DNA-dependent ATPase domain	69	81	57	75
Carboxyl terminus	66	79	34	56
***S. pombe* rhp54**				
Overall			50	69
Amino terminus			33	57
DNA-dependent ATPase domain			59	76
Carboxyl terminus			40	62

The deduced amino-acid sequence of the proteins shown in the table were aligned pairwise to calculate the percent of identical and similar amino-acid positions. The following amino acids were considered to be similar: D, E, N and Q; R, K and H; I, V, L and M; A, G, P, S and T; F, Y and W. No penalty was used to correct for gaps in the alignments. The amino termini of Rad54, rhp54 and hHR54 encompass amino acids 1–329, 1–288 and 1–177, respectively. The DNA-dependent ATPase domains encompass amino-acids 330–798, 289–752 and 178–638. The carboxyl terminus encompasses amino-acids 799–898, 753–852 and 639–747. Comparison of mHR54 with Rad54 and rhp54 gave results similar to the ones shown here for hHR54.

Figure 1



Comparison of the deduced amino-acid sequences of *S. cerevisiae* Rad54 [53], *S. pombe* rhp54 [21], mHR54 and hHR54. The complete amino-acid sequence of the proteins is shown in the single-letter code. Identical and similar amino acids to Rad54 in rhp54, mHR54 and hHR54 are shown in black and blue boxes, respectively. Dots denote gaps. Amino-acid positions are shown on the left. The seven motifs with similarity to the SNF2/SWI2 family are boxed [11]. The GenBank accession numbers for the cDNAs encoding mHR54 and hHR54 are X97796 and X97795, respectively.

levels of identity (Table 1). In particular, a potential nuclear localization signal was conserved in the amino terminus around amino- acid position 34 in the mammalian proteins.

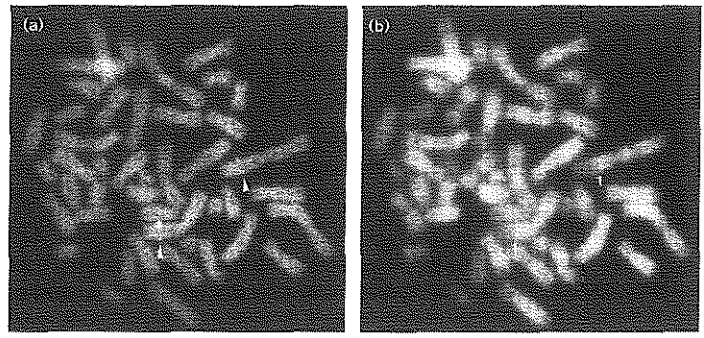
Chromosomal and subcellular localization

We determined the chromosomal localization of the *hHR54* locus to see whether it was located on a human chromosome that complements ionizing radiation-sensitive Chinese hamster ovary (CHO) cell lines. *In situ* hybridization showed that the *hHR54* cDNA localized to human chromosome 1 near band p32 (Fig. 2). This chromosome cannot correct the ionizing radiation-sensitive phenotype of the repair-deficient CHO cell lines tested to date [25]. Thus, it is unlikely that the *RAD54* homolog is defective in any of these CHO mutants.

The subcellular localization of the hHR54 protein was determined using polyclonal antibodies generated against a part of hHR54 produced in *E. coli* (see Materials and methods). COS-1 cells were transfected with a pSVL-derived expression vector and with the same vector containing the *hHR54* cDNA. In transfected cells, the hHR54 protein was detected in the nucleus by *in situ* immunofluorescence staining using affinity-purified anti-hHR54 antibodies (Fig. 3a,b). The staining was specific for hHR54 because no staining was observed when the pre-immune serum was used on COS-1 cells transfected with the *hHR54* expression vector (Fig. 3c). In addition, no staining was found in non-transfected COS-1 cells or in COS-1 cells transfected with the pSVL-derived vector itself (data not shown). The specificity of the affinity-purified anti-hHR54 antibody was also confirmed by immunoblot experiments. Protein extracts were prepared from HeLa cells and from the COS-1 cells that had been used in the immunofluorescence experiment. A band of the expected molecular weight was only detected in the COS-1 cells transfected

Figure 2

Chromosomal localization of *hHR54* by *in situ* hybridization. **(a)** Metaphase spreads of human chromosomes were hybridized to a biotinylated *hHR54* cDNA probe. The hybridization signal, indicated by the arrowheads, was detected on chromosome 1p32. **(b)** DAPI (4,6-diamidino-2-phenylindole) fluorescence of the chromosome spread shown in (a) to reveal the chromosomal banding pattern. Both chromosomes 1 are indicated.



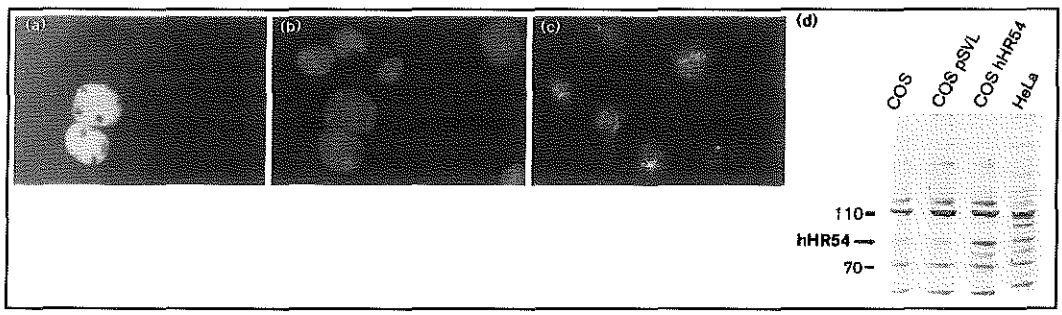
with the *hHR54* expression vector and in HeLa cells (Fig. 3d). However, the anti-hHR54 antibody was less specific under immunoblot conditions as compared to the immunofluorescence conditions, because some non-specific bands were observed on the immunoblot. We conclude that hHR54 resides in the nucleus. This observation is consistent with its potential nuclear localization signal and with a function of hHR54 in DNA metabolism.

Expression pattern of the mammalian *RAD54* homolog

We examined the expression of the mammalian *RAD54* homolog in different mouse tissues in order to determine where it might be active. The *mHR54* cDNA was used as a probe in northern blot analysis of total RNA from a variety of mouse tissues (Fig. 4a). Transcripts of *mHR54* were hardly detectable in most tissues. However, the level of the 2.6 kb *mHR54* transcript was dramatically increased in thymus, spleen and testis. The 18S ribosomal RNA indicated the relative amount of RNA loaded in each lane.

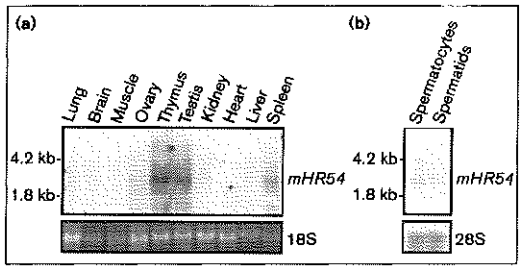
The increase in the *mHR54* transcript level in these tissues is consistent with a role of *mHR54* in V(D)J recombination — the process that brings together the 'variable', 'diversity' and 'joining' regions of antibody genes — and meiotic recombination. However, it cannot be excluded that the increased expression is simply a result of cellular

Figure 3



Subcellular localization of hHR54 by immunofluorescence staining. **(a)** hHR54 was detected in COS-1 cells transfected with a pSVL-derived expression vector containing the *hHR54* cDNA. Cells were fixed, stained with DAPI and treated with affinity-purified antibodies generated against part of hHR54 produced in *E. coli*. This primary antibody was detected with a goat anti-rabbit antibody coupled to fluorescin isothiocyanate (FITC). **(b)** DAPI fluorescence of the field of cells shown in (a) to identify the nuclei. **(c)** COS-1 cells transfected with the same hHR54 expression construct used in (a) and treated identically except that pre-immune serum was used as the source of primary antibodies. The photograph is highly overexposed compared to the one shown in (a). **(d)** Immunoblot from the cells used in the immunofluorescence experiment. Protein extracts were prepared from COS-1 cells, COS-1 cells transfected with the pSVL-derived vector itself (COS pSVL), COS-1 cells transfected with a pSVL derivative containing the hHR54 cDNA (COS hHR54) and HeLa cells. The extracts were electrophoresed through a 8.0 % SDS–PAGE gel, transferred to nitrocellulose, and probed with affinity-purified anti-hHR54 antibodies. Detection was with alkaline phosphatase-coupled goat anti-rabbit antibodies. The arrow indicates the position of hHR54. The size of the protein molecular weight markers (in kDa) is indicated on the left.

Figure 4

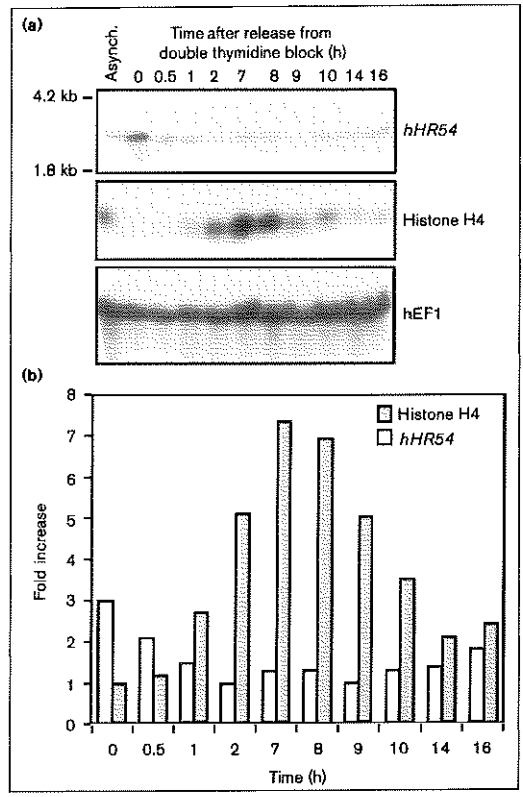


Expression of *mHR54* in different mouse tissues and during spermatogenesis. (a) Top panel: total RNA, isolated from the indicated mouse tissues, was electrophoresed through a 0.9 % formaldehyde–agarose gel, immobilized on nitrocellulose, and hybridized to a ^{32}P-labeled *mHR54* cDNA probe. Bottom panel: ethidium bromide-stained 18S ribosomal RNA in the gel used in the top panel to indicate relative amount of RNA loaded in each lane. (b) Top panel: total RNA, isolated from mouse spermatocytes and spermatids and analyzed as described above. Bottom panel: the membrane shown in the top panel was stripped and hybridized with a DNA probe derived from 28S ribosomal RNA to control for the amount of RNA loaded.

proliferation in the thymus, spleen and testis. To determine whether the increase in *mHR54* expression in testis correlated with meiosis we compared the level of *mHR54* mRNA in mouse spermatocytes and spermatids. Figure 4b shows a northern blot of total RNA isolated from meiotic spermatocytes and from post-meiotic spermatids [26]. The blot was probed with the *mHR54* cDNA; 28S ribosomal RNA was used as a loading control. Expression of *mHR54* was approximately 3-fold higher in spermatocytes compared to spermatids. In addition, two longer *mHR54*-derived transcripts seemed to be present only in spermatocytes. We conclude that increased expression of *mHR54* in testis coincides with the meiotic phase of spermatogenesis.

We next examined the regulation of *hHR54* expression during the cell cycle, as expression of *RAD54* in *S. cerevisiae* increases in late G1 phase [27]. HeLa cells were synchronized in late G1 by a double thymidine block [28]. Total RNA was isolated at different times after release from the block. The RNA was size-fractionated by gel electrophoresis, transferred to nitrocellulose, and sequentially hybridized with an *hHR54* cDNA probe, a histone H4 cDNA probe to identify S phase, and a human elongation factor 1 (hEF1) cDNA probe to control for RNA loading. Figure 5a shows the resulting autoradiograms. Quantitation of the *hHR54* and histone H4 mRNA levels is presented in Figure 5b. Expression of *hHR54* was increased approximately 3-fold in late G1 phase compared to other phases of the cell cycle. This increase was specific for *mHR54*, because no increase in late G1 phase was seen with hEF1 mRNA (Fig. 5a) and the transcripts of three

Figure 5



Determination of *hHR54* mRNA expression levels during the cell cycle. (a) HeLa cells were synchronized in late G1 phase by a double thymidine block. Total RNA was isolated from cells harvested at the indicated times after release from the second thymidine block, electrophoresed through a 0.9 % formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled *hHR54* cDNA probe (top panel). The first lane contains RNA isolated from a asynchronous population of HeLa cells. The membrane shown in the top panel was stripped and probed with a histone H4 cDNA to identify S phase (middle panel) and with a human elongation factor 1 (hEF1) cDNA to control for the amount of RNA loaded (bottom panel). (b) Quantitation of the autoradiograms shown in (a).

other DNA repair genes: *mHR51*, *mHR52* (data not shown) and the mouse homolog of *S. pombe rad21*$^+$ [29]. Thus, the regulation of the expression of *hHR54* and *RAD54* during the cell cycle seems to be conserved between yeast and humans.

Complementation of *S. cerevisiae rad54Δ* cells by *hHR54*

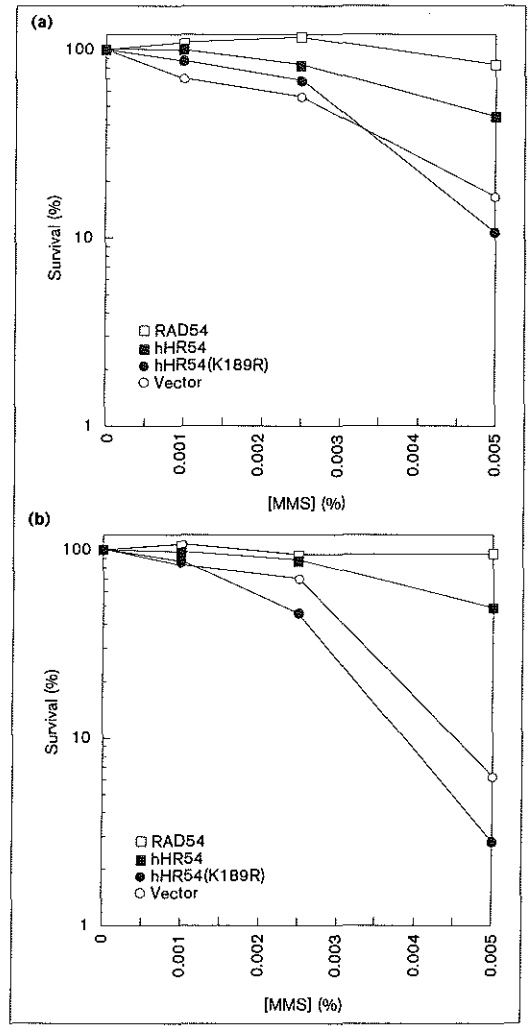
We next tested whether conservation of primary structure between Rad54 and hHR54 extended to conservation of

function. In *S. cerevisiae*, mutations in *RAD54* cause pleiotropic phenotypes in DNA damage repair, homologous recombination and meiosis because of a defect in double-strand break repair [3]. A role for *RAD54* in meiosis is suggested by the reduced spore viability of *rad54* mutants. A convenient DNA damage–repair phenotype to assess is sensitivity to MMS. We tested whether the MMS sensitivity of *S. cerevisiae rad54Δ* cells could be rescued by expression of the *hHR54* cDNA. Multicopy plasmids expressing either *RAD54* or the *hHR54* cDNA from the GAL10 promoter were transformed into *rad54Δ* cells. The expression vector served as a background control. Rescue was quantitated by determining the survival of the transformed cells in the presence and absence of MMS. Figure 6a shows that *rad54Δ* cells harboring the expression vector itself displayed a marked sensitivity to MMS; survival was reduced to 17 ± 13 % at 0.005 % MMS. In contrast, 83 ± 9 % of the *rad54Δ* cells containing the vector expressing *RAD54* survived at this MMS concentration. Expression of the *hHR54* cDNA resulted in partial complementation of the MMS-sensitive phenotype of the *rad54Δ* cells. In this case, 44 ± 12 % of the cells survived at 0.005 % MMS.

We determined whether the observed rescue depended on enzymatically active hHR54. An enzymatic activity that has been clearly demonstrated for proteins in the SNF2/SWI2 family is DNA-dependent ATPase activity [30,31]. Therefore, we tested whether the observed rescue depended on the ability of hHR54 to hydrolyze ATP. We changed the lysine residue at position 189 in the putative GKT Walker-type nucleotide-binding motif to an arginine residue by site-directed mutagenesis; this protein is referred to as hHR54(K189R). For a number of DNA helicases, including *E. coli* UvrD and *S. cerevisiae* Rad3, conversion of this invariant lysine residue into an arginine residue impairs NTP hydrolysis, while leaving nucleotide binding unaffected [32,33]. In addition, elimination of the ATPase activity of SNF2/SWI2 disrupts its function [31]. The cDNA encoding hHR54(K189R) did not rescue the MMS-sensitive *rad54Δ* phenotype. Only 11 ± 13 % of the cells survived at 0.005 % MMS; this was not significantly different from the vector control (Fig. 6a). An immunoblot confirmed that the wild-type and mutant proteins were expressed at similar levels in these experiments (data not shown). The equivalent of the K189R mutation in Rad54, K341R, also eliminated rescue of the MMS sensitivity of *S. cerevisiae rad54Δ* cells (J. Schmuckli and W-D.H., unpublished observations).

We next tested whether overexpression of hHR54 was required to rescue MMS sensitivity. The data shown in Figure 6a were obtained in the presence of galactose to induce the GAL10 promoter. The particular promoter we used gives a low level of gene expression when cells are grown on glucose (B.C. and W-D.H., unpublished observations).

Figure 6



Partial complementation of the MMS-sensitive phenotype of *S. cerevisiae* rad54Δ cells by *hHR54*. MMS sensitivity was determined by plating serial dilutions of transformant colonies onto selective plates with or without MMS (see Materials and methods). The percentage of colony forming units is plotted against the MMS concentration. *S. cerevisiae* strain FF18973 (rad54Δ) was transformed either with vector DNA (pWDH129), vector DNA containing RAD54 (pWDH195), vector DNA containing *hHR54* (pWDH213), or vector containing *hHR54(K189R)* (pWDH352). Shown are averages of four to six determinations. **(a)** Data obtained on media containing galactose; **(b)** data obtained using glucose as the carbon source. The standard deviations for the values at 0.005 % MMS for vector and *hHR54(K189R)* are overlapping. All other standard deviations at this concentration are not overlapping.

We therefore repeated the experiment shown in Figure 6a using glucose instead of galactose as a carbon source. Figure 6b shows that the complementation results under these conditions were similar to those obtained in the presence of galactose. The *hHR54* cDNA partially complemented the MMS sensitivity of *rad54Δ* cells as judged by a survival of 49 ± 10.9 % in 0.005 % MMS. The vector alone resulted in a survival of 6.2 ± 5.4 % at this MMS concentration. This is similar to the 2.8 ± 3 % survival measured for cells transformed with the *hHR54(K189R)* cDNA. Expression of *RAD54* increased survival to 95 ± 13 %. Thus, the partial complementation of the MMS-sensitive phenotype of the *rad54Δ* by *hHR54* does not require overexpression of *hHR54*. In addition, we found no dominant-negative effect of *hHR54* in wild-type *S. cerevisiae* cells, either under induced or non-induced conditions (data not shown). The data presented in Figure 6 show that *RAD54* and *hHR54* have a similar function in the repair of DNA damage inflicted by MMS.

Discussion

To safeguard their DNA from damage by environmental and endogenously generated genotoxins cells have developed a number of DNA repair pathways. Extensive studies on UV-sensitive *S. cerevisiae* mutants placed a number of genes involved in nucleotide excision–repair in the RAD3 epistasis group [34]. From analyses of the hereditary human disorder xeroderma pigmentosum, it has become clear that the RAD3 pathway is conserved during evolution [35]. Studies on ionizing radiation-sensitive *S. cerevisiae* mutants have shown that a number of genes involved in homology-dependent double-strand break repair are in the RAD52 epistasis group [1]. The recent isolation of several mammalian genes with similarity to the RAD52 group genes suggests that this repair pathway is also conserved. Here we describe the isolation of human and mouse cDNAs encoding hHR54 and mHR54, which have extensive amino-acid sequence similarity to *S. cerevisiae* Rad54; we also show that *hHR54* is a functional homolog of *RAD54* with respect to repair of MMS-induced DNA damage.

Rad54 and the mammalian proteins share seven motifs, indicated in Figure 1, which are referred to as the DNA-dependent ATPase domain. A number of superfamilies of proteins containing this domain have been identified [11]. Division of these large superfamilies places hHR54 and mHR54 in the SNF2/SWI2 family [11,12]. The sequence identity and similarity between Rad54 and hHR54 is highest within the ~460 amino-acid DNA-dependent ATPase domain (Table 1). However, it is likely that Rad54, hHR54 and mHR54 are homologs and not just different members of the SNF2/SWI2 family, because the sequence identity within their DNA-dependent ATPase domains is higher than that found among other family members. In addition, the amino and carboxyl termini contain a significant number of identical amino acids (Table 1). Similar levels of identity and similarity are found between the human and yeast homologs of *ERCC6*, which are SNF2/SWI2 family members involved in transcription-coupled nucleotide excision–repair [36].

Proteins in the SNF2/SWI2 family are involved in many aspects of DNA metabolism, including transcription, repair and recombination. The nuclear localization of hHR54 is consistent with a role for this protein in DNA metabolism (Fig. 3). The only enzymatic activity that has been demonstrated for proteins in the SNF2/SWI2 family is DNA-dependent ATPase activity [30,31]. It has been suggested that proteins within this subfamily are not DNA helicases, as is the case for some proteins within the superfamily, and that the conserved motifs that define the superfamily provide a function of which helicase activity is a subset [37]. A clear biochemical activity has been demonstrated for Mot1 from *S. cerevisiae*. This SNF2/SWI2 family member is capable of removing TATA-binding protein (TBP) from DNA, resulting in the global repression of RNA polymerase II transcription [30]. The protein complex containing SNF2/SWI2 and its human counterpart have been shown to help transcription factors overcome the repressive effects of chromatin, possibly by rearranging chromatin structure [31,38]. The biochemical activities of Rad54 and its mammalian homologs remain to be determined. DNA unwinding or chromatin-rearranging activities are attractive possibilities for proteins involved in homology-dependent double-strand break repair. Interestingly, other DNA repair pathways, such as nucleotide excision-repair and post-replication repair, also require at least one SNF2/SWI2 family member.

The existence of mammalian homologs of *RAD54* suggests that the RAD52 recombination pathway from *S. cerevisiae* is conserved in mammals. Of the *S. cerevisiae* genes that have been placed in the RAD52 epistasis group, four putative human homologs have now been identified. The amino-acid sequences of the human homologs of Rad51, Rad52, Rad54 and Mre11 are 67 %, 28 %, 48 % and 37 % identical to their *S. cerevisiae* counterparts, respectively ([15,19,22]; Table 1). Given the large evolutionary distance between yeast and humans this high degree of sequence identity strongly suggests conservation of the mechanism of homology-dependent double-strand break repair.

Using fluorescence *in situ* hybridization we localized the *hHR54* locus to chromosome 1p32 (Fig. 2). This chromosomal region has been implicated in the development of neuroblastoma. Tumor cells derived from 70 % of patients with neuroblastoma exhibit a deletion of part of the short arm of chromosome 1 [39]. Molecular analysis of a number of neuroblastoma cell lines indicates that the

deletions cluster at position 1p32 [40]. However, it remains to be determined whether *hHR54* plays a role in the development of neuroblastoma. The chromosomal position of the *hHR54* locus eliminates the possibility that any of the known ionizing radiation-sensitive CHO mutants, for which the correcting human chromosome has been identified, have a defect in its *hHR54* homolog, because none of these mutants can be complemented by chromosome 1 [25]. We tested six other ionizing radiation-sensitive CHO cell lines for correction of their sensitivity to the crosslinking agent mitomycin C by transfection of the *hHR54* cDNA. None of the lines tested (V-C8, V-H4, V4B3, V5B, V7B and V8B) could be corrected by the cDNA (data not shown).

Northern blot experiments showed that *mHR54* expression is increased in organs of lymphoid and germ cell development (Fig. 4a). The expression pattern of *mHR54* coincides with that of other mammalian RAD52 epistasis group genes. Expression of the mouse homolog of *RAD51* is increased in thymus, spleen and testis [15,16]. Expression of the *MRE11* homolog is elevated in spleen and testis [22]; expression in the thymus was not investigated in these experiments. Expression of the chicken and mammalian homologs of *RAD52* is increased in thymus and testis, but not in spleen [18,19]. The overlap in the expression pattern of these genes is consistent with a function of the encoded proteins in the same pathway. The increased expression of *mHR54* in thymus, spleen and testis is consistent with a role of *mHR54* in V(D)J and meiotic recombination. If *mHR54* is involved in V(D)J recombination, its role in this process is currently unclear. V(D)J recombination does not involve homologous recombination, but *mHR54* could mediate a substrate preparation step that V(D)J and meiotic recombination have in common, such as changing the chromatin structure of the loci that will be rearranged. A role for *mHR54* in meiotic recombination is suggested by its elevated expression in testis. To rule out the possibility that the increased *mHR54* expression in testis results from the high cell proliferation in this tissue and to better correlate *mHR54* expression with meiosis we determined *mHR54* mRNA levels during spermatogenesis. Expression of *mHR54* was approximately 3-fold higher in spermatocytes compared to spermatids (Fig. 5). This indicates that within testis tissue, *mHR54* expression is highest in cells undergoing meiosis.

In *S. cerevisiae*, expression of *RAD54* is cell-cycle regulated. Expression is increased approximately 4-fold in late G1-phase [27]. Because many genes required for S phase are highly expressed in late G1, *RAD54* could have a role in repairing DNA damage after replication [21,41]. We showed that the cell-cycle regulation of *hHR54* expression is similar to that of *RAD54*; *hHR54* is expressed throughout the cell cycle and its expression increases approximately 3-fold in late G1 phase (Fig. 5). Because V(D)J recombination seems to be initiated in G1 phase, our finding is consistent with a role for *hHR54* in processing the double-strand breaks involved in V(D)J recombination [42]. In contrast to the conservation of cell-cycle regulated expression between *RAD54* and *hHR54*, expression induced by ionizing radiation is not conserved, at least in primary fibroblasts. Although *RAD54* expression is induced by DNA-damaging agents such as MMS and γ rays, we found no increase in *hHR54* mRNA upon irradiation of primary fibroblasts with γ rays (data not shown). However, the relevance of *RAD54* induction is unclear, because *S. cerevisiae* strains that have lost *RAD54* inducibility are no more sensitive to DNA-damaging agents than the wild-type strain [43].

An important question that needs to be addressed when genes from different species are isolated based on amino-acid sequence conservation of the encoded proteins is whether these genes perform a similar function. Therefore, we tested whether *hHR54* could correct the DNA repair defect of *S. cerevisiae rad54Δ* cells. The repair defect in these cells is manifested by sensitivity to MMS. Figure 6 shows that expression of the *hHR54* cDNA does indeed complement the MMS sensitivity caused by deletion of *RAD54*. The final level of rescue by the *hHR54* cDNA is greater than 50 % of that achieved by expression of *RAD54*. The observed rescue depends on enzymatically active *hHR54*. A *hHR54* mutant, in which the lysine residue in the putative ATP-binding loop has been replaced by an arginine residue, is unable to rescue MMS sensitivity (Fig. 6). Based on biochemical studies, this mutation is likely to interfere with ATP hydrolysis [32,33].

Cross-species complementation has also been investigated for other RAD52 group genes. Reports on the rescue of MMS sensitivity of *S. cerevisiae rad51* mutants by the human and mouse *RAD51* homologs are conflicting. It seems that the human cDNA cannot complement a *rad51Δ* mutation [15]. However, the mouse cDNA might be able to suppress the repair defect of the *rad51-1* point mutation [16]. The *RAD52* homolog from chicken can partially rescue the MMS sensitivity and mating-type switching defects of the *S. pombe rad22-67* mutant (K. Ostermann and H. Schmidt, personal communication). Our complementation data show that *RAD54* and *hHR54* have a similar function in the repair of DNA damage inflicted by MMS. Taken together with the high conservation of *Rad54* and *hHR54* at the amino-acid level, it is likely that *hHR54* is indeed the functional homolog of *RAD54*.

It is informative to compare and contrast two fundamentally different mechanisms through which double-strand breaks can be repaired. In the yeast *S. cerevisiae*, double-strand breaks are repaired efficiently through a homology-dependent recombination mechanism [3]. From analyses

of cell lines deficient in double-strand break repair it seems that a homology-independent DNA end-joining mechanism plays a prominent role in mammals [25,44]. This apparent preference for different double-strand break repair mechanisms in yeast and mammals could have arisen because of their different genome organization. Repair by recombination ensures accurate restoration of sequences around the break, whereas repair by DNA end joining does not. In a unicellular organism such as *S. cerevisiae*, repair of double-strand breaks needs to be accurate to ensure survival, particularly because most of the *S. cerevisiae* genome contains coding information. Multicellular organisms such as mammals may not be critically dependent on accurate double-strand break repair, as mutations in their somatic cells may be tolerated more easily, especially given their large amount of non-coding DNA.

There is some evidence for two double-strand break repair pathways in mammalian cells [45]. Cell lines containing mutations in genes involved in DNA end joining are not completely deficient in the repair of double-strand breaks. In addition, mutant cell lines from the XRCC4 group show reduced sensitivity to ionizing radiation in late S–G2 phase [44]. It is possible that this reflects DNA repair through the homology-dependent recombination mechanism and requires the mammalian RAD52 group genes. Alternatively, the RAD52 group genes in mammals may have a more important function in meiosis than in the repair of double-strand breaks. These and other issues can be addressed when defined mutants in these mammalian genes become available.

Conclusions

The correct processing of double-strand breaks is an essential cellular process, both in germ cells during meiosis, and in somatic cells during repair of certain types of DNA damage. In *S. cerevisiae*, double-strand breaks are processed by the RAD52 recombination pathway. A number of genes similar to those in the RAD52 pathway have been isolated from mammals. For one of these genes, the *RAD54* homolog, we have shown here that this conservation of primary sequence extends to conservation of function.

We have provided three lines of evidence to show that *RAD54* has a mammalian homolog. First, mouse and human homologs are over 48 % identical to Rad54 from the yeasts *S. cerevisiae* and *S. pombe*. Given the large evolutionary distance between yeasts and mammals this degree of identity is highly significant. Second, the increase of *hHR54* and *RAD54* expression in late G1 phase is conserved. This cell-cycle regulated expression is shared with many genes required for S phase and is consistent with a function of *RAD54* and its mammalian homologs in post-replication repair [21,41]. Third, hHR54 can repair MMS-induced DNA damage in *S. cerevisiae* cells lacking Rad54. This result shows that hHR54 and Rad54 are functional homologs with respect to their DNA repair function. In addition, a role for *mHR54* in recombination is suggested by the demonstration that expression of the gene is essentially limited to organs with high recombination activity: thymus, spleen and testis. Moreover, increased expression in the testis coincided with the meiotic phase of spermatogenesis.

A number of DNA repair pathways, including the RAD3 nucleotide excision–repair pathway and the RAD52 recombinational repair pathway, require the close cooperation of many proteins to accurately execute the events leading to repair of DNA damage. It is clear that the RAD3 pathway is functionally conserved from yeast to humans. The observation that hHR54 functions in yeast cells suggests that this may also be the case for the RAD52 pathway. Therefore, our finding underscores the fundamental importance of DNA repair pathways — even though they involve multiple proteins, they seem to be functionally conserved throughout the eukaryotic kingdom.

Materials and methods

General procedures

Purification of nucleic acids, restriction enzyme digestions, gel electrophoresis, DNA ligation, synthesis of radiolabeled probes using random oligonucleotide primers, Southern, northern and western hybridizations, PCR, DNA sequencing and site-directed mutagenesis were all performed according to standard procedures [46]. Levels of mRNA were quantitated using a Molecular Dynamics phosphorimager.

Cell culture

COS-1 and HeLa cells were grown in F10–DMEM containing 5 % FCS. Transfection of plasmid DNA to COS-1 cells was carried out using the DEAE dextran method. HeLa cells were synchronized in late G1 phase by a double thymidine block as described [28].

Isolation of cDNAs encoding human and mouse homologs of RAD54

The following degenerate oligonucleotides were used to isolate a cDNA fragment of the chicken *RAD54* homolog: 5′–GGAATTC GAC/T CCI GAC/T TGG AAC/T CC and 5′–GGAATTC AA/G ATC/T TTC/T TCC/T TCI ATI GT. RT-PCR was performed as described [18]. Human and mouse testis cDNA libraries were hybridized at 61 °C in 3 × SSC, 10 × Denhardt's solution, 0.1 % SDS, 9 % dextran sulfate and 50 μg ml^{-1} salmon sperm DNA.

Accession numbers

The GenBank accession numbers for the cDNAs encoding mHR54 and hHR54 are X97796 and X97795, respectively.

In situ *hybridization*

Treatment of human lymphocyte metaphase spreads prior to hybridization was as described [47]. A *hHR54* cDNA fragment labeled with biotin was hybridized to the metaphase spreads as described [48]. Slides were incubated sequentially with 5 μg ml^{-1} avidin D–FITC (Vector, USA) and biotinylated goat anti-avidin D antibody, washed, dehydrated with ethanol and air-dried. Chromosomes were either counterstained with propidium iodide in antifade media or banded with DAPI and actinomycin D.

Immunofluorescent staining

A 1.72 kb *Hin*cII fragment from the *hHR54* cDNA was subcloned into the *Sma*I site of a pGEX-3X derivative. The fusion protein derived from this plasmid containing amino acids 146–684 of hHR54 fused to glutathione-S-transferase was produced in *E. coli* strain DH5α. The protein was purified from the insoluble fraction by preparative SDS–PAGE and used to immunize two rabbits. The resulting polyclonal antibodies were affinity purified using fusion protein immobilized on a nitrocellulose filter [49].

COS-1 cells were transfected with a pSVL (Pharmacia) derivative containing the *hHR54* cDNA to determine the subcellular localization of *hHR54*. As a negative control the pSVL-derived vector by itself was transfected to COS-1 cells. After 24 h the transfected COS-1 cells were transferred to slides for 24 h. The slides were sequentially incubated in PBS, PBS containing 2 % paraformaldehyde, and methanol. After washing in PBS containing 20 mM glycine and 5 mg ml^{-1} BSA the cells were incubated with primary antibodies. After washing, the cells were incubated with goat anti-rabbit FITC-conjugated antibodies. Slides were washed and preserved in Vectashield mounting media (Brunschwig). The DNA was stained with DAPI. Fluorescence microscopy was performed using an Aristoplan laser beam microscope.

Complementation of a S. cerevisiae rad54Δ *strain by* hHR54

The expression vector pWDH129, a derivative of pRDK249 [50], was used to direct the expression of *RAD54*, *hHR54*, or *hHR54(K189R)* in *S. cerevisiae* from the galactose-inducible GAL10 promoter. The *Cla*I–*Nhe*I *RAD54* fragment of Yep13-RAD54-216A [51] was ligated into the *Xho*I–*Nhe*I-digested pWDH129 together with a double-stranded oligonucleotide adapter composed of the sequences 5′–TCG AGA CAC CAT GGC AAG ACG CAG ATT ACC AGA CAG ACC ACC AAA TGG AAT AGG AG and 5′–CCG GCT CCT ATT CCA TTT GGT GGT CTG TCT GGT AAT CTG CGT CTT GCC ATG GTG TC, resulting in plasmid pWDH195. The *Dra*I–*Xba*I cDNA fragment of *hHR54* was cloned into the *Xho*I–*Nhe*I-digested pWDH129 after filling in the *Xho*I site using Klenow fragment of *E. coli* DNA polymerase I, resulting in plasmid pWDH213. The *hHR54(K189R)* mutation was constructed by oligonucleotide-directed mutagenesis. The *Xho*I–*Sac*I fragment encompassing the mutation was used to replace the corresponding fragment in pWDH213 to result in pWDH352.

Functional complementation of the *RAD54* deletion allele *rad54::LEU2* by vector DNA, *RAD54*, *hHR54*, and *hHR54(K189R)* was measured by determining the MMS sensitivity of strain FF18973 (*MATα, leu2-3 122, trp1-289, ura3-52, his7-2, lys1-1, rad54::LEU2*). Cell survival was determined in the presence and absence of increasing concentrations of MMS. All standard *S. cerevisiae* methods and media were as described [52]. Strain FF18973 was transformed with either pWDH129, pWDH195, pWDH213, or pWDH352 and after three days of incubation on SD-ura plates four to six independent transformants of each strain were picked into sterile water. 100 µl of each of four serial two-fold dilutions were plated on SD-ura plates with or without MMS, and with either 2 % galactose or 2 % glucose as the carbon source. Plates were incubated at 30 °C and colonies were counted after six days.

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CHAPTER 3

Genomic characterization and chromosomal localization of the mouse RAD54 gene

GENOMIC CHARACTERIZATION AND CHROMOSOMAL LOCALIZATION OF THE MOUSE *RAD54* GENE

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The yeast *Saccharomyces cerevisiae RAD54* gene belongs to the *RAD52* epistasis group whose members are involved in homologous recombination and DNA double-strand break repair. Recently, cDNAs encoding mouse and human homologs of yeast Rad54 have been isolated. Here we describe the isolation and characterization of the mouse *RAD54* genomic locus. The mouse *RAD54* gene spans a minimum of 30 kb and contains at least 18 exons. *In situ* hybridization experiments revealed that the gene maps to a region of chromosome 4 that is syntenic to human chromosome 1p, which contains the human *RAD54* gene.

INTRODUCTION

Extensive analyses of ionizing radiation sensitive yeast mutants established that DNA double-strand breaks (DSBs) are efficiently repaired through homologous recombination in the budding yeast *Saccharomyces cerevisiae*. Mutants in the so-called *RAD52* epistasis group are defective in DSB repair, resulting in sensitivity to ionizing radiation and methyl methanesulfonate [1]. In contrast, genetic analyses of ionizing radiation sensitive mammalian cell lines resulted in the identification of genes involved in DSB repair through DNA end-joining. The lack of mammalian mutant cells with a documented defect in homologous recombination contributed to the notion that DSB repair occurs via different mechanisms in yeast and mammals.

One of the key genes in homologous recombination in yeast is *RAD54*. Based on its primary amino acid sequence this gene belongs to the SNF2/SWI2 family of DNA-dependent ATPases. *S. cerevisiae* Rad54 (ScRad54) contains seven conserved motifs, including a Walker type A and B domain, found in a variety of proteins including the *E. coli* DnaB and RuvB proteins [2]. With the identification of the human and mouse homologs of ScRad54, it became possible to address the role of homologous recombination in mammals. In order to generate gene targeting constructs to disrupt mouse *RAD54* (*mRAD54*) in cells and mice, we have isolated the *mRAD54* genomic locus and examined its genomic organization.

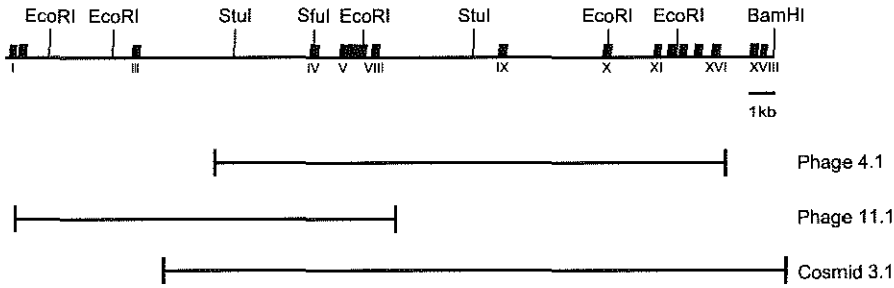

Figure 1. Characterization of the *mRAD54* genomic locus. Structure of the genomic *RAD54* locus and genomic phage and cosmid fragments hybridizing to the *mRAD54* cDNA. The top line represents the approximately 30-kb *mRAD54* genomic locus. Boxes indicate the 18 exons (I-XVIII) that make up the *mRAD54* cDNA. Shown are the locations of the selected restriction sites. Phage 4.1 and phage 11.1 cover most of the genomic *mRAD54* locus, except the 5' end which is covered by cosmid 3.1.

RESULTS AND DISCUSSION

The complete genomic locus encoding mRad54 was isolated from a lambda phage and a cosmid library. Two overlapping phage clones (phage 4.1 and phage 11.1) and one cosmid clone (cosmid 3.1), covering the *mRAD54* genomic locus were characterized in detail (Figure 1). In order to generate a restriction map of the *mRAD54* locus, *Eco*RI, *Hind*III and *Bam*HI single, double and triple digests of the positive phage and cosmid clones were hybridized to *mRAD54* cDNA. Hybridizing fragments were subcloned in plasmid vectors and sequenced to determine intron-exon borders. The length of the introns was determined by direct sequencing or PCR. The genomic organization of the *mRAD54* gene is depicted in Figure 1. The mRad54 protein is encoded by 18 exons ranging in size from 61 to 286 bp spread over a region of approximately 30 kb.

As shown in Figure 2, all sequences around intron-exon and exon-intron junctions are consistent with the consensus splice acceptor and donor signals. The first exon present in the *mRAD54* cDNA contains the coding sequence for the start codon immediately followed by a splice donor site. We cannot exclude the presence of additional untranslated exons upstream of exon 1. The first conserved motif (motif I) of the SNF2/SWI2 protein family, which corresponds to the Walkertype A box of the ATP-binding motif, and motif Ia are located in exon 7 of *mRAD54*. Motif II, corresponding to the Walker type B box of the ATP binding domain, lies in exon 8-9. The remaining conserved motifs are encoded by exon 9 (motif III), exon 14 (motif IV), exons 15-16 (motif V) and exon 16 (motif VI). Recently, the genomic organization of the human homolog of *ScRAD54 (hRAD54)* was reported [3]. The hRad54 protein is also encoded by 18 exons that span a minimum of 25 kb. The length and the relative position of the *mRAD54* and

hRAD54 exons and introns are remarkably conserved. For both the human and the mouse locus, introns 2-4, 8-10 and 16 are the largest introns. The first exon present in *hRAD54* cDNA, like the *mRAD54* contains the ATG start codon, immediately followed by a splice donor site.

INTRON SPLICE ACCEPTOR		EXON			INTRON SPLICE DONOR	
		I	(?)	90 GGATG	GTAAGTGTGA	(82 bp)
GCCTGGCTGCAGGATCACTGCAGGA ACTCACTTTCTGTTCTCCCTTGCAG	91 AGGAG	II	(87 bp)	176 CAGTA	GTGAGTGTTT	(4.5 kb)
TCTCTGGGGTTGTCTAGGTATTGCA ATATAGCTTTTCATTTCTCTCCTAG	177 ACTCC	III	(120 bp)	296 AACAT	GTAAGCCACA	(5.5 kb)
TCGAAGGTTAATAACATTTCAACAG TTTTGAATTTCTGTTTTCCTTTTAG	297 GAAGC	IV	(61 bp)	357 TCAAG	GTAAAATGGA	(1.2 kb)
GAATAAATAAATATAGAAGGTTGGC TTACAGGTTGGTTGCTGATTTCTAG	358 GTCCC	V	(137 bp)	494 GACAA	GTATCACATT	(350 bp)
TGAGTCTGATCATCATGGTTTTGTT TTGTTTTATCTGCTGTATTTCTCAG	495 GGAAA	VI	(70 bp)	564 GAGAG	GTAAAATGAG	(85 bp)
GCTGGGGGCTAAGTAGGCGCCAACA TGGACTAAATGGTGTTACTCATCAG	565 GGAGT	VII	(286 bp)	850 ACTGG	GTATGGAGCC	(220 bp)
GTTTTTCTTTAAGATCTTCTCATGT GTTCTCTGCAACCTTTCTGTTGTAG	851 AAGGA	VIII	(124 bp)	978 ACGAG	GTACTTAACA	(3.8 kb)
ATCGTTTAAGAATATGAAATCATCC TAAGTGAGACTTAATTGTCTTACAG	979 GGCCA	IX	(148 bp)	1127 TTTGG	GTAAGCAATT	(3.8 kb)
AAATCACCCTACCTTACATGCTTTC ATATTCAGTTCTCTCCTCTGTTTAG	1128 GAACT	X	(127 bp)	1255 AACAG	GTGAGAGGCT	(1.2 kb)
TTATTCTGCATCAGTTTCACCTCCC TTGTGCCTTTTCCCATTTCCCCTAG	1256 GTGCC	XI	(72 bp)	1328 TGTAG	GTATGAACTT	(150 bp)
TGTGTGCTCCAGTCTTTTCTATTGA GCTACACCTGCCCTTTTATTTTCAG	1329 GCTGA	XII	(139 bp)	1460 TAATC	GTGAGCCTCC	(550 bp)
AGTATGGTGGTGTTGCGCTTTTTTT TTTTTTTTCTTTTTTTGGTTTTCAG	1461 ATCCA	XIII	(90 bp)	1572 GTCAG	GTGAGCCTCC	(113 bp)
GGTTACTGGGATGACCAATTGTGAG GACTGATAATAGCCACTTTTCCCAG	1573 GTAAG	XIV	(138 bp)	1697 CGAAG	GTAGGGAAGA	(270 bp)
TTCCAATTAATTTATCGTTCTTGGG TCTTGATATTTGGATCTCCCTCAAG	1698 GTACT	XV	(80 bp)	1777 CATCG	GTAAGTATGT	(300 bp)
TTCCAAGTGTGTGCATCCTATGCTC TCCTTCTTGTTTCTTTTCTTCCCAG	1778 AGCCC	XVI	(182 bp)	1960 TATCT	GTAAGTATGT	(900 bp)
CCTCAGCATCTACAGGAATGGTTGA GTTCTACATTTCATCCCATTTCTAG	1961 GCAGG	XVII	(159 bp)	2120 GACAG	GTATGTGGAG	(90 bp)
GACCAAGCACAGAAATCCGTAGTGA CCACAACTCTGTCCTCTGACCCCAG	2121 ATTGC	XVIII				

Figure 2. Structural organization of the *mRAD54* gene. The nucleotide sequence of each intron-exon-intron junction is shown. The vertical lines represent the borders. All acceptor and donor sequences are in accordance with the consensus sequence $(Py)_nNCAG/G$ and (C,A)AG/GTPuAGT, respectively [8]. Exon numbering is as in Figure 1. The size of each intron and exon is given in parentheses.

We previously determined the genomic locus of *hRAD54* [4]. The *hRAD54* gene is located on human chromosome 1p32. Since this region is syntenic to both mouse chromosomes 3 and 4, it was important to determine the chromosomal localization of *mRAD54*. *In situ* hybridization experiments were performed on mouse metaphase spreads of an erythroid cell line carrying a trisomy of chromosome 4. The hybridization signal generated by the *mRAD54* probe showed that the *mRAD54* gene localizes to mouse chromosome 4, near band C7/D1 (Figure 3). Chromosome 4 was visualized using a mouse chromosome 4 specific paint probe.

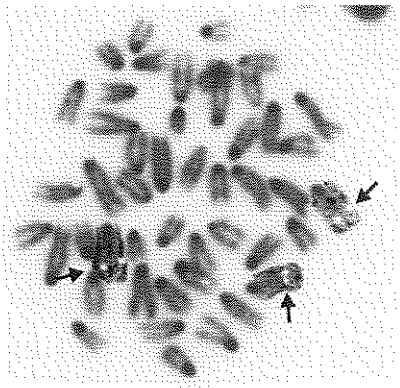

Figure 3. Chromosomal localization of *mRAD54* by *in situ* hybridization. Metaphase spreads of mouse chromosomes were hybridized to a digoxigenin-labeled *mRAD54* genomic probe and a biotinylated chromosome 4 specific paint probe. The hybridization signal, indicated by the arrowheads, was detected on chromosome 4, near band C7/D1. DAPI (4,6- diamidino-2-phenylindole) counterstaining of the chromosome spread reveals the chromosome banding pattern.

METHODS

General procedures. Purification of nucleic acids, digestion with restriction enzymes, DNA ligation, synthesis of radiolabeled probes using random oligonucleotide primers, Southern hybridizations, PCR, DNA sequencing were all performed according to standard procedures [5].

Identification of exon-intron borders. An *mRAD54* cDNA fragment was used to screen a lambda phage and a cosmid library made from a 129/Sv mouse strain (kindly provided by G. Grosveld and N. Galjart, respectively). Genomic fragments hybridizing to the *mRAD54* cDNA were subcloned in pBluescript II KS (Stratagene). The location of the intron-exon borders was determined by DNA sequencing, restriction site mapping and PCR analysis. Exon-intron borders were identified by the presence of consensus splice junctions at sites where the sequence of the genomic product differed from the *mRAD54* cDNA sequence. Intron size was determined by direct sequencing or by gel electroforesis of inter-exon PCR products (Expand Long Template PCR system, Boehringer).

***In situ* hybridization.** Treatment of mouse metaphase spreads of an erythroid cell line, containing a trisomy of chromosome 4 (code red8, N. J. de Both) prior to hybridization was as described [6]. A digoxigenin-labeled *mRAD54* genomic fragment and a biotin-labeled mouse chromosome 4 specific paint probe (Cambio) were denatured, competed with mouse Cot-1 DNA and hybridized to metaphase spreads as describeD [7]. To detect the *mRAD54* signal, slides were incubated sequentially with sheep-anti-digoxigenin-rhodamine and donkey-anti-sheep-texas-red. Chromosome 4 was detected using avidin D-FITC (Vector USA). Chromosome spreads were washed, dehydrated with ethanol, air-dried, and counterstained with DAPI in antifade media.

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CHAPTER 4

Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination

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Disruption of Mouse *RAD54* Reduces Ionizing Radiation Resistance and Homologous Recombination

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Summary

Double-strand DNA break (DSB) repair by homologous recombination occurs through the *RAD52* pathway in Saccharomyces cerevisiae. Its biological importance is underscored by the conservation of many *RAD52* pathway genes, including *RAD54*, from fungi to humans. We have analyzed the phenotype of mouse *RAD54*$^{-/-}$ (*mRAD54*$^{-/-}$) cells. Consistent with a DSB repair defect, these cells are sensitive to ionizing radiation, mitomycin C, and methyl methanesulfonate, but not to ultraviolet light. Gene targeting experiments demonstrate that homologous recombination in *mRAD54*$^{-/-}$ cells is reduced compared to wild-type cells. These results imply that, besides DNA end-joining mediated by DNA-dependent protein kinase, homologous recombination contributes to the repair of DSBs in mammalian cells. Furthermore, we show that *mRAD54*$^{-/-}$ mice are viable and exhibit apparently normal V(D)J and immunoglobulin class-switch recombination. Thus, *mRAD54* is not required for the recombination processes that generate functional immunoglobulin and T cell receptor genes.

Introduction

Double-strand DNA breaks (DSBs) are induced by ionizing radiation, which is often used in cancer therapy, and by radiomimetic agents including endogenously produced radicals. Accurate repair of these genotoxic lesions is essential for the prevention of chromosomal fragmentation, translocations, and deletions. All of these forms of genomic instability can lead to carcinogenesis through activation of oncogenes, inactivation of tumor suppressor genes, or loss of heterozygosity. Insight into the mechanisms of DSB repair in mammals is crucial for an understanding of the biological consequences of exposure to ionizing radiation.

Mutants in the Saccharomyces cerevisiae *RAD52* epistasis group are defective in DSB repair through homologous recombination and display sensitivity to ionizing radiation and the alkylating agent methyl methanesulfonate (MMS) (Game, 1993; Haber, 1995). Recently, it has become clear that the primary sequence of many *RAD52* group genes is conserved from yeast to humans (Petrini et al., 1997). Key genes in the *RAD52* DNA repair pathway include *RAD51* and *RAD54*. Biochemical experiments have demonstrated that Rad51 protein and its human counterpart, hRad51, are functional homologs of the Escherichia coli recombination protein RecA. These proteins form nucleoprotein filaments with single-stranded DNA and mediate homologous DNA pairing and strand exchange (Ogawa et al., 1993; Benson et al., 1994; Sung, 1994; Sung and Robberson, 1995; Baumann et al., 1996; Gupta et al., 1997). Genetic experiments with yeast have revealed that Rad54 and its human counterpart, hRad54, are functional homologs with respect to the repair of MMS-induced DNA damage (Kanaar et al., 1996). The proteins belong to the SNF2/SWI2 family of DNA-dependent ATPases (Eisen et al., 1995). Its members have been implicated in the remodeling of chromatin structure in the context of many aspects of DNA metabolism such as transcription, recombination, and repair (Kingston et al., 1996). Rad54 might function together with Rad51 since genetic and physical interactions between these two S. cerevisiae proteins have been observed (Jiang et al., 1996; Clever et al., 1997).

Mechanisms of mammalian DSB repair have been investigated with the use of a collection of ionizing radiation–sensitive Chinese hamster cell lines (Jeggo, 1990). A number of these cell lines have specific defects in DSB repair, and the correcting genes have been identified. Genetic and biochemical analyses have revealed that these cell lines are defective in DNA end-joining that requires no or extremely limited DNA homology (Jeggo et al., 1995; Roth et al., 1995). Proteins involved in this pathway include the subunits of DNA-dependent protein kinase (DNA-PK): the DNA-PK catalytic subunit (DNA-PK$_{cs}$) and the Ku70/80 heterodimer, which has DNA end-binding activity. In contrast to the plethora of yeast mutants, there are no mammalian mutant cell lines with documented defects in homologous recombination. Therefore, while the contribution of homologous recombination to ionizing radiation resistance in yeast is well established, its contribution in mammals is unknown.

Recently, it has been established that many of the proteins involved in DNA end-joining are also required for immunoglobulin and T cell receptor (TCR) gene rearrangement (Jeggo et al., 1995; Roth et al., 1995). Two types of DNA rearrangements lead to the production of functional immunoglobulin genes during B cell development. The first is V(D)J recombination, which determines the antigenic specificity of the encoded antibody. It occurs at recombination signal sequences and is initiated by a site-specific DSB introduced by the RAG1 and RAG2 proteins (Gellert, 1996). Additional proteins required for V(D)J recombination include DNA-PK and XRCC4 (Gellert, 1996). Functional TCR genes are also assembled through V(D)J recombination. The second DNA rearrangement is immunoglobulin class-switch recombination, which changes the constant region of the immunoglobulin heavy (H) chain, thereby switching the isotype of the encoded antibody (Harriman et al., 1993).

While the early immune response in mice is dominated by expression of immunoglobulin M (IgM) and IgD, class-switch recombination allows expression of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE. Class-switch recombination is more reminiscent of homologous recombination than of site-specific recombination, because it involves switch regions that vary between 2 and 10 kb and contain a large array of short repeated sequences. In contrast to V(D)J recombination, enzymatic activities required for class-switch recombination have not yet been identified. Since the expression of all mammalian *RAD52* group genes, isolated to date, is increased in organs of lymphoid development, it is of considerable interest to determine whether they are involved in immunoglobulin and TCR gene rearrangements.

To address whether recombinational repair contributes to ionizing radiation resistance in mammals, we have generated mouse embryonic stem (ES) cells in which both alleles of *mRAD54* have been disrupted. These cells have a reduced level of homologous recombination as measured by gene targeting. This observation provides genetic evidence for the functional conservation of the *RAD52* homologous recombination pathway in mammalian cells. Significantly, *mRAD54*$^{-/-}$ cells display sensitivity to ionizing radiation, mitomycin C, and MMS compared to otherwise isogenic wild-type and heterozygous mutant cell lines. These results suggest that besides DNA end-joining, homologous recombination contributes to ionizing radiation resistance in mammalian cells. To assess the biological impact of homologous recombination at the level of the intact organism, we generated *mRAD54*$^{-/-}$ mice. In contrast to inactivation of the *mRAD51* gene, which causes an embryonic lethal phenotype (Lim and Hasty, 1996; Tsuzuki et al., 1996), *mRAD54*$^{-/-}$ mice are viable. Analyses of cell surface markers, indicative of V(D)J recombination, on cells isolated from thymus, bone marrow, peritoneal cavity, and spleen have not revealed a difference between *mRAD54*$^{+/+}$ and *mRAD54*$^{-/-}$ mice. In addition, the level of different immunoglobulin subclasses in the serum of mutant mice is similar to that found in wild-type mice. These results show that *mRAD54* is not required for V(D)J and immunoglobulin class-switch recombination.

Results

Generation of *mRAD54*-Disrupted Mouse Cells

We generated mouse ES cells lacking mRad54 to determine the phenotype of a mammalian *RAD54* mutant. Using the *mRAD54* cDNA as a probe, the *mRAD54* genomic locus was isolated by screening a lambda and a cosmid library made from strain 129/Sv genomic DNA. Three clones that spanned the approximately 30 kb *mRAD54* genomic locus (Figure 1A) were obtained. The locus was extensively characterized by restriction site mapping and sequencing of intron–exon borders. The EcoRI fragment containing exon 4 was subcloned and disrupted by insertion of selectable marker genes at a position corresponding to nucleotide position 307 in the *mRAD54* cDNA. The insertion results in elimination of 90% of the protein, including all seven highly conserved motifs implicated in the essential DNA-dependent ATPase activity. Moreover, aberrant RNA splicing that skips the targeted exon would result in a frameshift mutation. Three disruption constructs containing the neomycin-, hygromycin-, and puromycin-selectable markers were made. They are referred to as *mRAD54*^{307neo}, *mRAD54*^{307hyg}, and *mRAD54*^{307pur}, respectively.

The *mRAD54*^{307neo} construct was electroporated into E14 ES cells. After selection, targeted clones were identified by DNA blotting using a unique probe outside the targeting construct (Figure 1B). Multiple independent cell lines containing one disrupted *mRAD54* allele were obtained at a frequency of approximately 40%. The second wild-type allele was disrupted by targeting with either the *mRAD54*^{307hyg} or the *mRAD54*^{307pur} construct. Several double-knockout cell lines in which the remaining wild-type allele was replaced by *mRAD54*^{307hyg} or *mRAD54*^{307pur}, respectively, were obtained. In addition, heterozygous mutant cell lines in which the targeting constructs had replaced the previously targeted *mRAD54*^{307neo} allele instead of the wild-type allele (Figure 1B) were obtained. No significant difference in growth rate between *mRAD54*$^{+/+}$ ES cells and all of the different *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ ES cell lines was detected (data not shown). We conclude that, similar to the situation in S. cerevisiae and Schizosaccharomyces pombe (Muris et al., 1996), disruption of the *RAD54* homolog in mouse cells results in viable cells.

We next investigated the consequence of disrupting *mRAD54* at the protein level. The *mRAD54* cDNA encodes a protein of 747 amino acids with a predicted molecular weight of 84.6 kDa. The human homolog of mRad54 is 94% identical and migrates at this predicted molecular weight (Kanaar et al., 1996; data not shown). A protein of the expected molecular weight was detected in extracts from HeLa cells and from wild-type and *mRAD54*$^{+/-}$ mouse ES cells by immunoblotting using affinity-purified anti-hRad54 antibodies (Figure 1C). In contrast, the protein could not be detected in *mRAD54*$^{-/-}$ ES cells. The nature of the 110 kDa protein that cross-reacted with the anti-hRad54 antibodies is unknown. In addition, RNA blotting experiments showed that the *mRAD54* transcript, though present in *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ ES cells, was not detectable in *mRAD54*$^{-/-}$ ES cells (data not shown). We conclude that disruption of both copies of *mRAD54* exon 4 results in an *mRAD54* null mutation.

mRAD54$^{-/-}$ ES Cells Are Sensitive to Ionizing Radiation and MMS

S. cerevisiae *RAD52*-group mutants, including *rad54*Δ, are sensitive to ionizing radiation and MMS. We therefore investigated the effect of these DNA-damaging agents on *mRAD54*$^{-/-}$ ES cells. They were found to be approximately 2.3-fold more sensitive to γ-irradiation than *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ cells (Figure 2A). In this experiment and all others described in this article, no significant difference was found between wild-type and heterozygous mutant cells. In addition, *mRAD54*$^{-/-}$ cells were found to be 3- to 4-fold more sensitive to MMS than *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ cells (Figure 2B). These results are consistent with the notion that S.

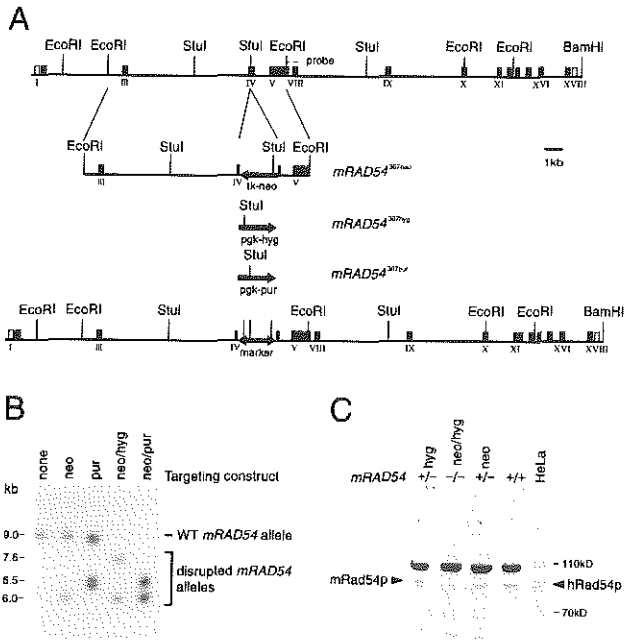

Figure 1. Characterization of the *mRAD54* Genomic Locus and Generation of *mRAD54*-Disrupted Mouse ES Cells

(A) Structure of the genomic *mRAD54* locus and gene targeting constructs. The top line represents the approximately 30 kb *mRAD54* genomic locus. All 18 exons that make up the *mRAD54* cDNA are indicated by boxes. The exons containing the start and stop codons are represented by open boxes. The dashed line above exons 7 and 8 indicates the position of the probe used to screen for the disrupted allele. Shown are the locations of selected restriction sites. The middle line represents the targeting constructs and shows the position of the selectable marker genes neomycin (*neo*), hygromycin (*hyg*), and puromycin (*pur*) in exon 4. The disrupted genomic locus is represented by the bottom line. The vertical lines in the double-headed arrow indicate the relative position of the diagnostic StuI site in the three marker genes. (B) DNA blot of *mRAD54* wild-type and mutant ES cells. ES cells were electroporated with the three different targeting constructs. Genomic DNA was isolated from individual clones and digested with StuI. Clones containing the homologously integrated targeting constructs were identified by DNA blotting using the probe indicated in (A). Heterozygous mutant cell lines containing the $mRAD54^{307neo}$ allele were subsequently targeted with the $mRAD54^{307hyg}$ and $mRAD54^{307pur}$ targeting constructs to generate $mRAD54^{-/-}$ cell lines.

(C) Immunoblot of protein extracts from *mRAD54* wild-type and mutant ES cells. Protein extracts from $mRAD54^{-/-}$, $mRAD54^{-/-}$, $mRAD54^{-/-}$, and HeLa cells were separated on an 8% SDS–polyacrylamide gel electrophoresis gel, transfered to nitrocellulose, and hybridized with affinity-purified anti-hRad54 antibodies. Detection was performed with alkaline phosphatase–coupled goat anti-rabbit antibodies. The sizes of the protein molecular weight markers are indicated in kilodaltons on the right. The arrowheads indicate the position of the mammalian Rad54 proteins.

cerevisiae *RAD54* and *mRAD54* are functional homologs (Kanaar et al., 1996). We conclude that disruption of *RAD54* in S. cerevisiae and in mouse ES cells results in a qualitatively similar phenotype with respect to ionizing radiation and MMS sensitivity.

$mRAD54^{-/-}$ ES Cells Are Sensitive to Mitomycin C but Not to Ultraviolet Light

Mitomycin C introduces interstrand cross-links in DNA (Tomasz et al., 1987). We tested $mRAD54^{-/-}$ cells for sensitivity to this agent because DSBs are likely intermediates in the repair of such lesions. $mRAD54^{-/-}$ cells were found to be 2- to 3-fold more sensitive to mitomycin C than $mRAD54^{+/+}$ and $mRAD54^{+/-}$ cells (Figure 3A). To prove that the phenotype of the $mRAD54^{-/-}$ cells was caused exclusively by the disruption of *mRAD54*, cDNA rescue experiments were performed. $mRAD54^{307neo/hyg}$ cells were electroporated with a cDNA construct expressing mRad54 together with a plasmid expressing the dominant selectable marker puromycin. Puromycin-resistant colonies were characterized by DNA blotting and immunoblot analysis to identify cell lines that stably expressed mRad54 (data not shown). The cDNA construct fully corrected the mitomycin C sensitivity of $mRAD54^{-/-}$ ES cells (Figure 3A). To show that the $mRAD54^{-/-}$ ES cells were specifically deficient in recombinational DNA repair, we tested the sensitivity of the cells to ultraviolet (UV) irradiation, which causes damage that is repaired primarily by nucleotide excision repair. No significant difference in sensitivity was detected among $mRAD54^{+/+}$, $mRAD54^{+/-}$, and $mRAD54^{-/-}$ ES cells (Figure 3B).

Homologous Recombination in $mRAD54^{-/-}$ ES Cells Is Reduced

It is generally believed that homologous recombination, resulting in targeted integration, is relatively rare in mammalian cells compared to illegitimate recombination, resulting in random integration. However, it has previously been demonstrated that homologous rather than illegitimate recombination can be predominant in mammalian cells (te Riele et al., 1992). To test whether *mRAD54* is involved in homologous recombination in mouse ES cells, we performed gene targeting experiments. *mRAD54* wild-type, heterozygous mutant ($mRAD54^{307neo/+}$), and double-knockout ($mRAD54^{307neo/hyg}$) cells were electroporated with a construct designed to target the mouse *CSB* locus (van der Horst et al., 1997). This targeting construct is referred to as *CSB-pur* since it contains a phosphoglycerate kinase (PGK) promoter-driven puromycin resistance gene as a dominant selectable marker. The total number of puromycin-resistant colonies obtained was similar for each genotype (see legend to Table 1). Genomic DNA was isolated

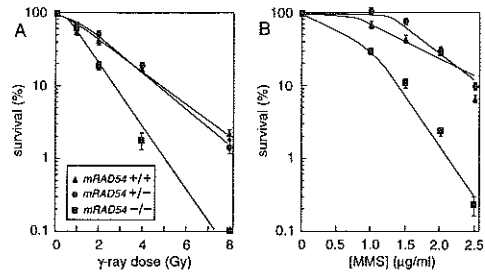

Figure 2. Effect of Ionizing Radiation and MMS on Wild-type and Mutant *mRAD54* ES Cells

(A) Clonogenic survival of *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ ES cells after treatment with increasing doses of γ-rays. After irradiation, cells were plated and allowed to form individual colonies. The percentage of surviving cells as measured by their colony-forming ability is plotted as a function of the γ-ray dose. For all survival experiments described in this article, cells were grown for 7–10 days after treatment and subsequently fixed, stained, and counted. Cloning efficiencies varied from 10% to 30%. All measurements were performed in triplicate. Consistent results were obtained among different sets of experiments. The mutant cells used in the survival experiments were *mRAD54*$^{307neo/+}$ and *mRAD54*$^{307neo/hyg}$.

(B) Clonogenic survival of *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ ES cells after treatment with increasing concentrations of MMS. Fresh serial dilutions of MMS were prepared in PBS and added to the growth medium for 1 hr, after which the cells were washed twice with PBS and returned to fresh growth medium.

from individual clones and analyzed by DNA blotting to discriminate between homologous and random integration events. Targeting efficiency was measured as the percentage of clones containing the homologously integrated targeting construct relative to the total number of analyzed drug-resistant clones (Table 1). The targeting efficiency of the *CSB-pur* construct was 18% and 20%

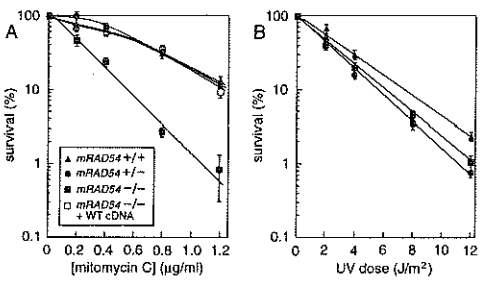

Figure 3. Effect of Mitomycin C and UV Light on Wild-type and Mutant *mRAD54* ES Cells

(A) Clonogenic survival of *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ ES cells after treatment with increasing concentrations of the cross-linking agent mitomycin C and rescue of the mitomycin C sensitivity of *mRAD54*$^{-/-}$ cells with an *mRAD54* cDNA expression construct. This survival experiment was performed as described for the MMS treatment. An *mRAD54*$^{-/-}$ cell line containing randomly integrated *mRAD54* cDNA expression constructs was obtained as described in Experimental Procedures. Expression of mRad54 in this cell line was verified by immunoblot experiments.

(B) Clonogenic survival of *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ ES cells after treatment with increasing doses of UV light.

Table 1. Homologous Recombination Is Reduced in *mRAD54*$^{-/-}$ ES Cells: Percentages of Homologous Integration Events among Total Number of Integration Events

	Targeting Construct		
ES Cell Genotype	*CSB-pur*	*RB-pur*	*RB-hyg*
mRAD54$^{+/+}$	18% (10/56)	ND	ND
mRAD54$^{neo/+}$	20% (19/95)	20% (19/96)	13% (10/76)
mRAD54$^{neo/hyg}$	4% (3/69)	3% (2/61)	NA
mRAD54$^{neo/pur}$	NA	NA	<1% (0/82)

ES cells of the indicated genotype were electroporated with the indicated targeting constructs. The frequency of drug resistant clones obtained for each genotype was similar. It varied from 2 to 5×10^{-4} for *CSB-pur*, from 2.5 to 3.5×10^{-4} for *RB-pur*, and from 3 to 6×10^{-5} for *RB-hyg*. Values shown are the percentage of clones containing homologously integrated targeting construct relative to the total number of analyzed clones; absolute numbers are given in parentheses. No significant difference in the frequency of homologous integration of *CSB-pur* was observed in the *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ cell lines ($p > 0.5$ by chi-squared analysis). In contrast, this frequency was significantly different between those cell lines and the *mRAD54*$^{-/-}$ cell line ($p < 0.01$). The frequency between the *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ cell lines for *RB-pur* and *RB-hyg* was also significantly different ($p < 0.01$ and $p < 0.005$, respectively). ND, not determined; NA, not applicable.

in *mRAD54*$^{+/+}$ and *mRAD54*$^{307neo/+}$ cells, respectively. In contrast, only 4% of the *mRAD54*$^{307neo/hyg}$ puromycin-resistant colonies contained homologously integrated *CSB-pur* DNA.

To exclude the possibility that the reduction of homologous recombination in *mRAD54*$^{-/-}$ cells was due to the nature of the *CSB* locus, we performed additional targeting experiments to the mouse *RB* locus, which is unrelated to the *CSB* locus. An *RB-pur* construct had a targeting efficiency of 20% in heterozygous mutant *mRAD54* cells (Table 1). This efficiency was identical to the one found for *CSB-pur*. Significantly, homologous integration of the *RB-pur* construct was reduced approximately 7-fold in *mRAD54*$^{-/-}$ cells. In addition, the targeting efficiency of a second *RB* targeting construct (*RB-hyg*) containing the hygromycin resistance gene as selectable marker was determined in an independently obtained *mRAD54*$^{-/-}$ cell line (*mRAD54*$^{307neo/pur}$). The targeting efficiency of this construct was more than 10-fold reduced in the *mRAD54*$^{-/-}$ cell line compared to the *mRAD54*$^{+/-}$ control cell line.

In summary, the gene targeting experiments showed that homologous recombination was reduced in two different targeting loci and in two independently generated *mRAD54*$^{-/-}$ cell lines. These experiments provide genetic evidence for involvement of *mRAD54* in homologous recombination in mammalian cells. Interestingly, homologous recombination was not completely inactivated in *mRAD54*$^{-/-}$ cells.

Generation of *mRAD54*-Deficient Mice

The experiments described above show that absence of mRad54 is compatible with normal ES cell growth. Subsequently, we investigated whether mRad54 is required for normal mouse development. Two of the targeted ES clones, carrying the *mRAD54*^{307neo} allele, were injected into C57BL/6 blastocysts. One clone transmitted the disrupted *mRAD54* allele to the mouse germline.

Two chimeras transmitted the coat color encoded by the ES cell genome to almost all of their 65 offspring, of which 29 were heterozygous for the disrupted *mRAD54* allele. F1 heterozygous offspring were intercrossed, and F2 offspring were genotyped by DNA blotting or polymerase chain reaction (PCR) analysis or both (data not shown). Among 52 genotyped animals, 12 *mRAD54*$^{+/+}$, 29 *mRAD54*$^{+/-}$, and 11 *mRAD54*$^{-/-}$ animals were identified. This outcome is consistent with a normal Mendelian segregation of the disrupted *mRAD54* allele. Thus, an *mRAD54* null mutation does not result in embryonic or neonatal lethality. No statistically significant difference in weight was observed among *mRAD54*$^{-/-}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{+/+}$ littermates. Importantly, the *mRAD54*$^{-/-}$ mice exhibited no macroscopic abnormalities up to at least 5 months of age.

mRad54 Protein Is Not Required for V(D)J Recombination

To investigate whether *mRAD54* is necessary for the processing of DSBs during V(D)J recombination, we examined the expression of immunoglobulin and TCR genes in the *mRAD54*$^{-/-}$ mice by flow cytometry. In the thymus, the total quantity of cells, the CD4/CD8 profile, and the expression of TCR$\alpha\beta$ or TCR$\gamma\delta$ were normal (Figure 4). In addition, no accumulation of immature Thy-1$^+$/Il-2R$^+$ thymocytes, as occurs in RAG1- or RAG2-deficient mice (Mombaerts et al., 1992; Shinkai et al., 1992), was observed in *mRAD54*$^{-/-}$ mice, and normal quantities of CD4$^+$ and CD8$^+$ cells were present in the spleen (data not shown). Thus, TCR expression and the differentiation process leading to the generation of mature single-positive T cells occurs efficiently in the absence of mRad54.

B cell development was studied by analysis of surface expression of B220, CD43, IgM, and IgD, which resolve B cell progenitors into discrete stages (Hardy et al., 1991). No differences among *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ mice were observed in the various B cell progenitors in the bone marrow. The transition of B220$^+$CD43$^+$ pro-B cells to B220$^+$CD43$^-$ pre-B cells, which marks the completion of functional immunoglobulin H V(D)J recombination, is not affected by the absence of mRad54 (Figure 4). In the peritoneal cavity the quantity of conventional B cells and CD5$^+$ B cells was in the normal range (data not shown). In addition, the quantity of B cells in the spleen and their IgM/IgD profiles were normal (Figure 4). These results show that mRad54 is not essential for V(D)J recombination.

mRad54 Protein Is Not Required for Immunoglobulin H Chain Class-Switch Recombination

B cells can join the V(D)J exon to different downstream effector H chain constant regions by recombination between repetitive switch regions present 5′ of each germline H chain constant region. To investigate whether *mRAD54* is involved in the immunoglobulin H chain class-switch recombination from IgM to IgG, IgA and IgE, we determined the levels of immunoglobulin subclasses in the serum using an enzyme-linked immunosorbent assay (ELISA). The concentrations of IgM as well as the concentrations of secondary isotypes, including

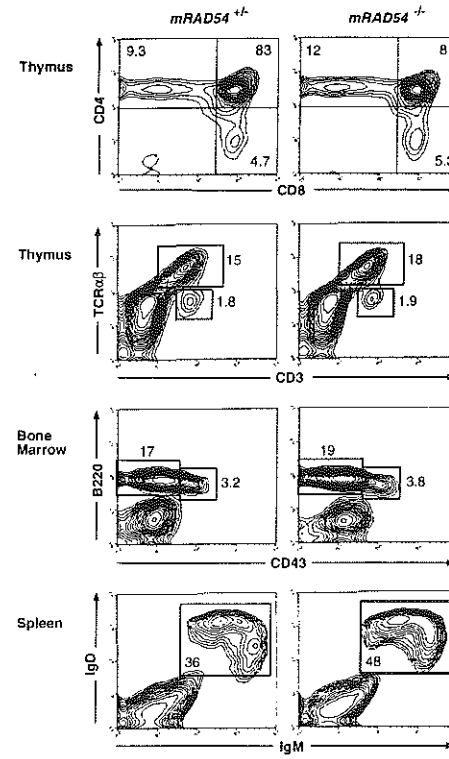

Figure 4. Analysis of V(D)J Recombination in *mRAD54* Wild-type and Mutant Mice

Flow cytometric analysis of indicated tissues in 5-week-old *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice. For thymus cell suspensions, the percentages of cells that express CD4 and/or CD8, as well as the percentages of CD3$^+$TCR$\alpha\beta^+$ and CD3$^+$TCR$\alpha\beta^-$ (i.e., TCR$\gamma\delta^+$) cells, are indicated. Bone marrow cells were stained with anti-IgM, anti-B220, and anti-CD43. IgM$^-$ B cell progenitors are displayed, and the percentages of total bone marrow cells that are B220$^+$CD43$^+$ pro-B cells and B220$^+$CD43$^-$ pre-B cells are indicated. For spleen cell suspensions the percentages of IgM$^+$IgD$^+$B220$^+$ cells are given. Shown are representative experiments from analyses of six *mRAD54*$^{-/-}$ and six control mice (*mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$). Data are shown as 5% probability contour plots. Dead cells were gated out based on forward and side scatter characteristics. The x and y axes are logarithmic, spanning values from 10^0 to 10^4.

IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE, were indistinguishable among *mRAD54*$^{+/+}$, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ mice (Figure 5). These results show that *mRAD54*$^{-/-}$ cells are proficient in immunoglobulin isotype switching.

Discussion

The importance of the *RAD52* DNA repair pathway in the yeast S. cerevisiae has been well established. The pathway is pivotal in maintaining genomic stability by accurately repairing DSBs through homologous recombination (Game, 1993; Haber, 1995). In stark contrast, the contribution of homologous recombination to the

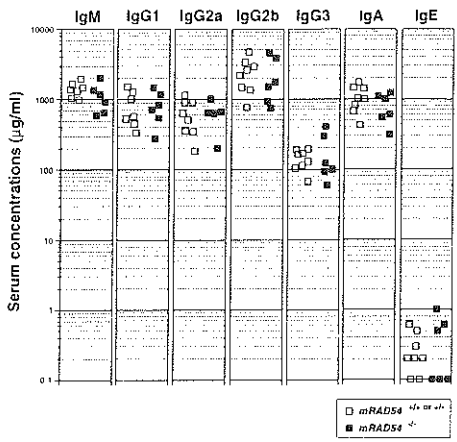

Figure 5. Analysis of Immunoglobulin Class Switch Recombination in *mRAD54* Wild-type and Mutant Mice

Serum concentrations of immunoglobulin subclasses in unimmunized 2-month-old mice, determined by sandwich ELISA. Five mice had IgE levels below the detection limit of the assay (0.1 μg/ml). Six *mRAD54*$^{-/-}$ mice were analyzed; the control group consisted of three *mRAD54*$^{+/+}$ and five *mRAD54*$^{+/-}$ mice.

repair of DNA damage in mammals is currently unclear. The recent isolation of human and mouse genes with similarity to the *RAD52* group genes suggests that this DNA repair pathway is conserved in mammals. In this article, we report our use of reverse genetics to show that the mouse *RAD52* group member, *mRAD54*, is involved in homologous recombination and contributes to the repair of certain types of DNA damage. Similar results have been obtained with the use of a chicken-derived cell line (Bezzubova et al., 1997 [this issue of *Cell*]). Together, these results provide compelling genetic evidence for the functional conservation of the *RAD52* homologous recombination pathway from yeast to mammals.

The Phenotype of *mRAD54*$^{-/-}$ ES Cells Is Consistent with a Defect in DSB Repair

S. cerevisiae strains carrying mutations in the *RAD52* group genes are sensitive to ionizing radiation. *RAD51*, *RAD52*, and *RAD54* form a distinct subset because mutations in these genes result in higher X-ray sensitivity than mutations in the other *RAD52* group genes. In addition, they have been most convincingly implicated in DSB repair (Game, 1993). Analysis of the effect of the *RAD52* group genes in mammalian cells requires mutant cell lines. To date, however, only targeted disruption of a single *mRAD51* allele in mouse ES cells has been described. Homozygous null mutants could not be isolated, either by growing *mRAD51*$^{+/-}$ cells under elevated drug concentrations or by targeting the *mRAD51* alleles with two different selectable markers (Lim and Hasty, 1996; Tsuzuki et al., 1996). These results suggest that *mRAD51*$^{-/-}$ cells are inviable because either mRad51-dependent DNA repair is essential in mammals or the mammalian *RAD51* gene has an additional, yet unidentified, essential function. A number of additional *recA* analogs have been identified in yeast, and they are likely to exist in mammals as well. If these analogs do exist in mammals, they apparently are unable to take over the essential function of mammalian *RAD51*.

Here, we demonstrate that in contrast to *mRAD51*$^{-/-}$ cells, *mRAD54*$^{-/-}$ cells are viable (Figure 1). Disruption of one or both *mRAD54* alleles has no negative effect on the growth rate and cloning efficiency of the cells. In all of the experiments performed to date, no phenotype of heterozygous mutant *mRAD54* cells has been detected. In contrast, *mRAD54*$^{-/-}$ cells are sensitive to ionizing radiation and MMS (Figure 2). These phenotypes are similar to those of S. cerevisiae *rad54*Δ cells. *mRAD54*$^{-/-}$ cells display a sensitivity to ionizing radiation that is quantitatively similar to that of mouse *ATM*$^{-/-}$ ES cells (Xu and Baltimore, 1996). *ATM*$^{-/-}$ ES cells contain a disruption in the mouse homolog of the human *ATM* gene, which is responsible for the ataxia telangiectasia syndrome (Meyn, 1995). Whereas *RAD54* is likely to be involved directly in the repair of DNA damage, *ATM* is probably required indirectly, by mediating proper cell cycle arrest upon the induction of DNA breaks (Meyn, 1995). An abnormal G1 checkpoint function in *ATM*$^{-/-}$ cells is reflected by the increased level of radioresistant DNA synthesis in these cells (Barlow et al., 1996). In contrast, we observed no increase in radioresistant DNA synthesis in *mRAD54*$^{-/-}$ cells, suggesting that this checkpoint is not affected by the absence of mRad54 (data not shown).

Analyses of DSB repair-deficient mammalian cell lines have shown that DNA end-joining, involving DNA-PK, plays a prominent role in repair of ionizing radiation-induced DNA damage (Jeggo, 1990; Jeggo et al., 1995). Defective DNA-PK$_{cs}$ activity is associated with the mouse *scid* mutation (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995). The *scid* mutation confers a 2- to 3-fold radiosensitivity in mice and their derived tissues and cells (Biedermann et al., 1991). A similar severity in ionizing radiation sensitivity is seen in *mRAD54*$^{-/-}$ ES cells, which appear to be impaired in homology-dependent DNA repair (see below). Interestingly, *mRAD54*$^{-/-}$ cells are sensitive to mitomycin C (Figure 3), while mouse cells containing the *scid* mutation are not mitomycin C sensitive (Biedermann et al., 1991; Hendrickson et al., 1991). One form of DNA damage introduced by mitomycin C is an interstrand cross-link. An intermediate in the repair of these lesions is believed to be a DSB. Thus, although both DNA end-joining and homologous recombination pathways can contribute to the repair of DSBs, specialized roles for either pathway may exist (see below). Now that mutants in both repair pathways are available, the specific roles of the different DSB repair pathways in mammals can be clarified.

mRAD54 Is Involved in Homologous Recombination in Mammalian Cells

The involvement of the *RAD52* group genes in homologous recombination in S. cerevisiae and S. pombe has been clearly established. Homologous integration in S.

cerevisiae is approximately 8-fold reduced in *rad51* strains and is virtually eliminated in *rad52* strains compared to wild-type controls (Schiestl et al., 1994). S. pombe strains containing disrupted *RAD51* and *RAD54* homologs are approximately 15-fold reduced in homologous integration, while mutations in the *RAD52* homolog reduce homologous integration 2-fold (Muris et al., 1997). In general, homologous recombination in mammalian cells is relatively rare compared to random integration. The use of an isogenic targeting construct is considered to be one of the most important requirements for efficient homologous integration in mammals because DNA mismatch repair suppresses homeologous recombination (te Riele et al., 1992; de Wind et al., 1995). With the use of isogenic targeting constructs, we have shown that *mRAD54* is required for efficient homologous integration in mouse ES cells. In its absence, homologous integration is 5- to 10-fold reduced (Table 1). These results imply that gene targeting in mammalian cells can occur through a *RAD52*-like recombination pathway.

mRAD54-Deficient Mice Are Viable

To determine the biological effects of mutations in mammalian *RAD52* group genes at the whole-animal level, mice containing disrupted *RAD52* group genes are invaluable. It is difficult to uncover these effects in *mRAD51*$^{-/-}$ mice because they have an early embryonic lethal phenotype (Lim and Hasty, 1996; Tsuzuki et al., 1996). In contrast, the absence of *mRAD54* is compatible with normal mouse development. Interbreeding of F1 *mRAD54* heterozygous mutant mice results in F2 offspring in which all three genotypes are detected in a Mendelian ratio. This proves that disruption of *mRAD54* does not interfere with embryonic or neonatal development. *mRAD54*$^{-/-}$ mice are healthy, without gross abnormalities, up to at least 5 months of age. Although mRNA expression of all isolated mammalian *RAD52* group genes is increased in testis, *mRAD54*$^{-/-}$ mice produce offspring (data not shown). This shows that *mRAD54* has no essential function in meiosis, although more subtle meiotic defects have to be analyzed in more detail.

V(D)J Recombination and Class Switch Recombination Occur in *mRAD54*-Deficient Mice

The mammalian germline genome does not contain functional immunoglobulin and TCR genes. Instead, they are generated through rearrangements of gene segments during B and T cell development. In mice and humans, functional immunoglobulin and TCR genes are generated through V(D)J recombination. V(D)J recombination is initiated by a site-specific DSB, and a number of genes involved in DNA end-joining take part in processing this DSB (Jeggo et al., 1995; Gellert, 1996). Consequently, B and T cell development is arrested at an early stage in mice homozygous for a disruption of the DNA-PK component Ku80 (Nussenzweig et al., 1996; Zhu et al., 1996). In contrast, B and T cell development is normal in *mRAD54*$^{-/-}$ mice (Figure 4). This result implies that V(D)J recombination can take place efficiently under conditions of reduced homologous recombination.

In addition to V(D)J recombination, immunoglobulin genes can undergo class-switch recombination, which controls antibody isotype. In contrast to V(D)J recombination, immunoglobulin class-switch recombination is more reminiscent of homologous recombination since it occurs between regions containing repetitive sequences (Harriman et al., 1993). However, the recombination break points are not always within the repetitive sequences (Dunnick et al., 1993). A possible relationship between homologous recombination and immunoglobulin class-switch recombination is suggested by immunolocalization experiments. Anti-hRad51 antibody staining appears to be confined to mouse B cells, switching from expression of IgM to IgG antibodies (Li et al., 1996). However, as Figure 5 shows, the concentration of antibodies of all major isotypes is similar in the serum of *mRAD54*$^{+/+}$ and *mRAD54*$^{-/-}$ mice. These results imply that mRad54 does not play a major role in immunoglobulin class-switch recombination.

The analysis of the immune system of *mRAD54*$^{-/-}$ mice presented in this article shows that immunoglobulin gene rearrangements can take place in the absence of mRad54. A role for the mammalian *RAD52*-group genes in the development of the immune system was suggested by the observation that all of these genes are highly expressed in organs of lymphoid development, such as thymus and spleen. We show here that *mRAD54* is not required for immunoglobulin and TCR gene rearrangements. Our results do not exclude the possibility that *mRAD54* function is redundant (see below) or that the absence of mRad54 results in subtle effects on the exact sequences of joints between the various immunoglobulin and TCR gene segments.

Possible Redundancy of *mRAD54* Function

At present it is unclear whether there is redundancy in any of the functions of *mRAD54*. Duplication of DNA repair genes during evolution is not uncommon. For example, there are at least two human and mouse homologs of the yeast DNA repair genes *RAD6* and *RAD23*. In addition, the *RAD52*-group genes *RAD55* and *RAD57* show sequence similarity to *RAD51*, and overexpression of *RAD51* suppresses DNA repair phenotypes of *rad55* and *rad57* cells (Hays et al., 1995; Johnson and Symington, 1995). Genes with some similarity to *RAD54* have been identified in yeast and humans (Stayton et al., 1994; Eki et al., 1996). They are clearly not as closely related to *RAD54* as are *hRAD54* and *mRAD54*, and no functional data on these genes are available. The repair and recombination phenotypes of *RAD54*-deficient S. cerevisiae and mouse cells demonstrate that some functions of *RAD54* are not redundant. However, although homologous recombination is reduced in *mRAD54*$^{-/-}$ cells, it is not completely absent. In addition, we have not yet uncovered an obvious meiotic phenotype of *mRAD54*$^{-/-}$ mice. These observations suggest that *mRAD54* homologs with specialized functions might exist.

Besides redundancy of *mRAD54* function, there could be redundancy in DNA repair pathways. The same end point may be reached by different repair pathways

through distinct mechanisms. For example, a role for DNA end-joining in the repair of ionizing radiation–induced DNA damage has been clearly demonstrated, and we show here that homologous recombination also contributes to ionizing radiation resistance. The two DNA repair pathways might differ in tissue, cell cycle, and/or damage specificity since they may have different properties. In this respect, the pathways are likely to differ in the accuracy of repair. DNA end-joining might not result in accurate repair since nucleotides at the break could be added or lost and incorrect ends might be joined. In contrast, homologous recombination ensures accurate repair because the undamaged sister chromatid or homologous chromosome is used as a template. While accurate repair seems critical for germ and stem cells, inaccurate repair could be more easily tolerated by differentiated somatic cells given that a large fraction of their genome is no longer functional.

The *RAD52* Homologous Recombination Pathway Is Functionally Conserved from Yeast to Mammals

The accumulated evidence for the functional conservation of the *RAD52* pathway in mammals is now extremely compelling. First, both Rad51 and Rad54 are highly conserved from yeast to mammals, despite their large evolutionary distance. Second, key steps in homologous recombination—the search for homology and strand exchange—are mediated in vitro by the purified yeast and human Rad51 proteins (Benson et al., 1994; Sung, 1994; Sung and Robberson, 1995; Baumann et al., 1996; Gupta et al., 1997). Third, human *RAD54* can partially complement certain DNA repair phenotypes of S. cerevisiae *rad54Δ* cells (Kanaar et al., 1996). Fourth, mouse *RAD54*$^{-/-}$ cells have a qualitatively similar phenotype compared to S. cerevisiae *rad54Δ* cells with respect to ionizing radiation and MMS sensitivity (Figure 2). Fifth, mouse *RAD54*$^{-/-}$ cells are impaired in homologous recombination (Table 1). Since *mRAD54*$^{-/-}$ mice are viable, they provide an experimental mouse model with a defined defect in a gene implicated in homology-dependent DSB repair and permit detailed assessment of the importance of this process for genetic stability, oncogenesis, and mitotic and meiotic recombination repair.

Experimental Procedures

Construction of *mRAD54* Targeting Vectors

An *mRAD54* cDNA fragment was used to screen a lambda phage and a cosmid genomic library made from a 129/Sv mouse strain (kindly provided by G. Grosveld and N. Galjart, respectively). Genomic fragments hybridizing to the *mRAD54* cDNA were subcloned in pBluescript II KS (Stratagene). The location of the intron–exon borders was determined by DNA sequencing, restriction site mapping, and PCR analysis. An approximately 9 kb EcoRI fragment encompassing exons 4, 5, and 6 and containing a unique SfuI restriction site in exon 4 was subcloned in pBluescript II KS. Three targeting vectors were made. The first was made by inserting a cassette with the neomycin resistance gene driven by the TK promoter in the unique SfuI site of exon 4. The resulting *mRAD54* allele is refered to as *mRAD54*^{307neo}. A second targeting vector was made by inserting a PGK hygromycin expression cassette in the unique exon 4 SfuI site (*mRAD54*^{307hyg}), and a third targeting vector was made by inserting a PGK puromycin cassette in the SfuI site (*mRAD54*^{307pur}).

ES Cell Culture and Electroporation

E14 ES cells (kindly provided by A. Berns, The Netherlands Cancer Institute, Amsterdam) were electroporated with the *mRAD54*^{307neo} targeting construct and cultured on gelatinized dishes as described (Zhou et al., 1995). G418 was added 24 hr after electroporation to a final concentration of 200 μg/ml, and the cells were maintained under selection for 6–8 days. Genomic DNA from individual G418-resistant clones was digested with StuI and analyzed by DNA blotting using a flanking probe. Targeted clones with the expected hybridizing StuI fragments were subsequently screened with a fragment of the neomycin resistance gene to confirm proper homologous integration. To obtain ES cell lines carrying a disruption in both *mRAD54* alleles, an *mRAD54*^{307neo}-targeted ES cell line was electroporated with the *mRAD54*^{307hyg} targeting construct. After selection with hygromycin B (200 μg/ml) for 10 days, colonies were isolated and expanded. Using the same flanking probe as was used in the *mRAD54*^{307neo} targeting experiment, cell lines containing the homologously integrated *mRAD54*^{307hyg} construct in either the wild-type or the previously targeted *mRAD54* allele were obtained. In a similar experiment with the *mRAD54*^{307pur} targeting construct, *mRAD54*$^{307neo/pur}$ double-knockout and *mRAD54*$^{307pur/+}$ single targeted cell lines were obtained. Clones were selected with puromycin (1 μg/ml) for 10 days.

Cell Survival Curves

The sensitivity of ES cells to increasing doses of DNA-damaging agents was determined by measuring their colony-forming ability. ES cells were trypsinized and counted. Various cell dilutions were aliquoted onto gelatinized 60 mm dishes, and after 12–24 hr, cells were incubated 1–2 hr in drug-containing media. Ionizing radiation sensitivity was determined by comparing the colony-forming ability of targeted ES cells after irradiation with an ^{137}Cs source. For UV irradiation (254 nm), the medium was replaced with PBS prior to exposure. Cells were grown for 7–10 days, fixed, stained, and counted. All measurements were performed in triplicate.

Rescue of *mRAD54*$^{-/-}$ Cells by cDNA Expression

The *mRAD54* cDNA was subcloned in pPGK-p(A). This cDNA expression construct was coelectroporated with a PGK puromycin plasmid in *mRAD54*$^{307neo/hyg}$ cells. Clones were selected with puromycin (1 μg/ml) for 10 days. Integration of the cDNA construct and mRad54 expression were confirmed by DNA blotting and immunoblot analysis, respectively.

Homologous Targeting of *RB* and *CSB* Loci

Targeting and subsequent analysis of the *RB* and *CSB* loci in E14, *mRAD54*$^{+/-}$, and *mRAD54*$^{-/-}$ ES cells were done as described previously (Zhou et al., 1995). The targeting constructs *129RB-hyg* and *129RB-puro* were kindly provided by H. te Riele (The Netherlands Cancer Institute, Amsterdam). The targeting construct *CSB-pur* was obtained by inserting a PGK puromycin expression cassette in an unique XhoI site of an approximately 7 kb genomic fragment of the mouse *CSB* gene (van der Horst et al., 1997). Targeted integration in the *RB* and *CSB* loci was distinguished from random integration by DNA blot analysis using appropriate probes flanking the targeting constructs (data not shown).

Generation of *mRAD54* Mutant Mice

Cells of *mRAD54*^{307neo}-targeted clones were karyotyped, and ES cells from clones with 40 chromosomes were used for injection into C57BL/6 blastocysts (Zhou et al., 1995). Male chimeric mice were mated with C57BL/6 females and found to transmit the disrupted *mRAD54* allele to the germline. *mRAD54*$^{-/-}$ mice were obtained by intercrossing F1 heterozygotes. Genotyping was performed by DNA blot analysis with a flanking probe or PCR analysis or both.

Flow Cytometric Analysis

Single-cell suspensions from lymphoid tissues were prepared and stained with monoclonal antibodies, and 3×10^4 cells were scored with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) (Hendriks et al., 1996). Antibodies obtained from Pharmingen (San

Diego, CA) included phycoerythrin (PE)–conjugated anti-CD4, anti-CD25, anti-CD43, anti-CD5, biotinylated anti-IgM, anti-CD8, fluorescein isothiocyanate–conjugated anti-B220/RA3-6B2, and anti-CD3. Phycoerythrin-conjugated anti-IgD was purchased from Southern Biotechnology Associates (Birmingham, AL). Purified monoclonal antibodies anti-Thy1/59AD22, anti-TCRαβ/H57597, and anti-TCRγδ/GL3 were conjugated to biotin using standard procedures. TriColor-conjugated streptavidin (Caltag Laboratories, CA) was used as a secondary antibody.

Serum Immunoglobulin Detection

Levels of immunoglobulin subclasses in serum were measured by sandwich ELISA using unlabeled anti-mouse immunoglobulin subclass-specific antibodies (Southern Biotechnology) as capture reagents. Serially diluted sera were incubated for 3 hr. Subsequently, peroxidase-labeled anti-mouse immunoglobulin subclass-specific antibodies and azino-bis-ethylbenz-thiazoline-sulfonic acid were added.

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CHAPTER 5

DNA damage sensitivities of RAD54 knockout mice

DNA DAMAGE SENSITIVITIES OF *RAD54* KNOCKOUT MICE

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DNA double-strand breaks (DSBs) and interstrand DNA crosslinks interfere with key steps in DNA metabolism such as replication and transcription. Repair of these very genotoxic lesions, essential for the maintenance of genome integrity, can occur through homologous recombination [1]. We have shown previously that disruption of the mouse *RAD54* (*mRAD54*) gene results in embryonic stem (ES) cells that are defective in homologous recombination and are sensitive to ionizing radiation (IR) and the DNA crosslinking agent mitomycin C [2]. To better define the role of *mRAD54*-mediated homologous recombination in DNA damage repair in other cell types, we have generated *RAD54*-deficient mice. Unexpectedly, we find that these mice are not IR sensitive, but do retain the sensitivity to mitomycin C. Our results suggest that an alternative DSB repair pathway plays a more dominant role in repairing IR-induced DNA damage in differentiated cells compared to pluripotent ES cells. In contrast, the important contribution of Rad54-dependent homologous recombination towards repair of interstrand DNA crosslinks remains in at least some differentiated cell types.

INTRODUCTION

Mutants in the *Saccharomyces cerevisiae RAD52* epistasis group display hypersensitivity to IR and are defective in DSB repair through homologous recombination [3, 4]. Key proteins in the *RAD52* epistasis group are Rad51, Rad52, and Rad54. Biochemical analyses have shown that the central events of recombination, homologous DNA pairing and strand exchange are mediated by Rad51 [5] and that both Rad52 and Rad54 can stimulate the recombination activities of Rad51 [6-9]. The functional significance of DSB repair through homologous recombination is underscored by the conservation of the *RAD52* pathway from fungi to humans. Mammalian homologs of *RAD51*, *RAD52* and *RAD54* have been identified [10, 11] (and Chapter 1) and human Rad51 and Rad52 have been shown to possess similar activities as their yeast counterparts [12, 13]. While homologous recombination is the predominant DSB repair pathway in bacteria and yeast, DNA end-joining is believed to be the principal DSB repair pathway in mammalian cells

[14, 15] (Chapter 1). DNA end-joining is mediated by, among others, DNA-dependent protein kinase, XRCC4 and DNA ligase IV [16] (Chapter 1).

We have shown that the human Rad54 (hRad54) is a functional homolog of yeast Rad54 [17] (Chapter 2). Therefore, to address the role of *mRAD54*-mediated homologous recombination in mammals, we generated ES cells, in which both alleles of the *mRAD54* gene have been disrupted by gene targeting. Consistent with a DSB repair defect, *mRAD54*$^{-/-}$ ES cells are sensitive to IR and mitomycin C. Gene targeting experiments demonstrated that homologous recombination was reduced 5 to 10-fold compared to wild-type ES cells [2]. Similar results were described for a *RAD54*$^{-/-}$ mutant of the chicken DT40 cell line [18]. Since both ES cells and the chicken DT40 cells are believed to exhibit unusually high ratios of targeted to random integration after DNA transfection, we wanted to determine the contribution of Rad54-dependent homologous recombination to repair of DNA damage in more differentiated cells or in a whole organism.

mRAD54$^{-/-}$ mice provide an excellent opportunity to study the significance of the mammalian *RAD52* pathway. In contrast to homozygous disruption of other genes implicated in DSB repair, such as *mRAD51*, *mMRE11*, *BRCA1* and *BRCA2*, which results in an embryonic lethal phenotype [19-22], *mRAD54*$^{-/-}$ mice are viable [2]. *mRAD54*$^{-/-}$ mice display no gross abnormalities and have a normal life expectancy. Therefore, *mRAD54*$^{-/-}$ mice enable us to compare IR and mitomycin C sensitivity among different cell types and compare them to those of the same cell types derived from wild-type mice.

RESULTS AND DISCUSSION

We first tested whether the observed IR sensitivity of *mRAD54*$^{-/-}$ ES cells extended to *mRAD54*$^{-/-}$ mice. Four groups of five *mRAD54*$^{-/-}$ and five *mRAD54*$^{+/-}$ mice were irradiated with doses of 6, 7, 7.5 and 8 Gy, respectively. Unexpectedly, only one *mRAD54*$^{-/-}$ mouse irradiated with 7 Gy, died within three weeks. Irradiation with 8 Gy was lethal for all mice, regardless of *mRAD54* status. Next, we addressed whether the lack of IR sensitivity of *mRAD54*$^{-/-}$ mice could be due to their genetic background. The *mRAD54*$^{-/-}$ ES cells are derived from a 129 mouse strain, while the *mRAD54*$^{-/-}$ mice are hybrids derived from C57Bl6 and 129 mice. It is possible that the difference in genetic background between the ES cells and the mice masks the effect of *mRAD54* disruption. To test this hypothesis, we isolated *de novo* ES cells from the *mRAD54*$^{-/-}$ C57Bl6/129 mice. A *RAD54*-proficient control cell line was generated by introduction of a cDNA expressing hRad54 into the *mRAD54*$^{-/-}$ C57Bl6/129 ES cell line. Interestingly, the 129- and C57Bl6/129- derived *mRAD54*$^{-/-}$ ES cells showed similar sensitivities to IR compared to their *mRAD54*-proficient control cells (Figure 1A). We conclude that *mRAD54*$^{-/-}$ mice are not sensitive to IR and that this lack of IR sensitivity compared to that of *mRAD54*$^{-/-}$ ES cells is not due to a difference in genetic background.

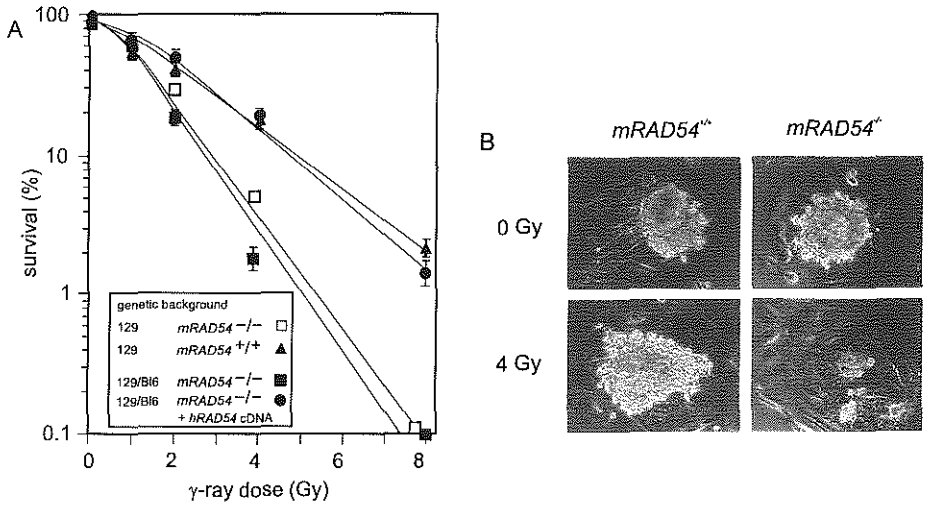

Figure 1. Effect of IR on ES cells and day 3.5 embryos. **A**, Effect of IR on 129 and C57Bl6/129 *mRAD54*-proficient and -deficient ES cells. The percentage of surviving cells as measured by their colony-forming ability is plotted as a function of IR dose. The 129 *mRAD54*-deficient ES cells were generated by gene targeting [2]. The C57Bl6/129 *mRAD54*-deficient ES cells were isolated *de novo*. A *RAD54*-proficient derivative of this ES cell line was generated by selecting a cell line that stably expressed a *hRAD54* cDNA construct. **B**, After isolation, day 3.5 embryos were irradiated with a dose of 0 or 4 Gy of IR and photographed 10 days after culture. Outgrowth of the inner cell mass and trophoblast cells from embryos was determined.

Next, we determined whether, in contrast to the adult stage, *mRAD54*-deficiency resulted in IR sensitivity at the embryonic stage. We isolated *mRAD54*$^{-/-}$ and *mRAD54*$^{+/+}$ embryos at day 3.5 of gestation. Embryos were treated with doses of 0 or 4 Gy of IR and subsequently cultured *in vitro* for 10 days (Figure 1B). Without irradiation the percentage of embryos showing outgrowth of the inner cell mass and trophoblast cells was similar for both mutant and control embryos (Table 1). Inner cell mass outgrowth was slightly reduced for control embryos after exposure to 4 Gy. However, after 10 days the inner cell mass of all thirteen irradiated *mRAD54*$^{-/-}$ embryos was completely ablated. The presence of trophoblast cells provided a control for initial attachment of the embryo to the culture dish. Thus, in contrast to adult *mRAD54*$^{-/-}$ mice, *mRAD54*$^{-/-}$ embryos are sensitive to IR.

To examine whether *mRAD54*-deficient cells derived from a late embryonic stage showed DNA damage sensitivities similar to those observed for *mRAD54*$^{-/-}$ ES cells, we isolated mouse embryonic fibroblasts (MEFs). Established MEF cell lines were derived from the primary MEFs after multiple passages of these cells. A control Rad54-proficient cell line was generated by transfecting the *mRAD54*$^{-/-}$ MEFs with a cDNA expressing hRad54. Cell lines stably expressing hRad54 were identified by immunoblotting (data not shown). We showed previously that hRad54

Outgrowth of inner cell mass from day 3.5 embryos		
Dose (Gy)	Genotype	
	mRAD54$^{+/+}$	*mRAD54*$^{-/-}$
0	100% (3/3)	93% (14/15)
4	77% (10/13)	< 7% (0/13)

Table 1. Quantitation of the results shown in Figure 1B. *mRAD54*-proficient and -deficient embryos were treated with an IR dose of 0 or 4 Gy. The percentage of embryos showing outgrowth of the inner cell mass was obtained by dividing the number of embryos showing outgrowth of the inner cell mass and the trophoblast cells by the number of embryos showing outgrowth of the trophoblast cells only after 10 days of *in vitro* culture. Absolute numbers are shown in parentheses. Attachment of trophoblast cells was used as an internal control.

complements the IR and mitomycin C sensitivity of *mRAD54*$^{-/-}$ ES cells [23]. The *mRAD54*$^{-/-}$ MEFs were found to be 1.4-fold more sensitive to IR than their Rad54-proficient derivatives (Figure 2 and Table 2). mRad54-deficiency resulted in a 2-fold sensitivity towards mitomycin C (Figure 2 and Table 2). Thus, the IR and mitomycin C sensitivity of *mRAD54*$^{-/-}$ MEFs is less than that of *mRAD54*$^{-/-}$ ES cells.

We previously demonstrated that homologous recombination is reduced in *mRAD54*$^{-/-}$ ES cells [2]. To test whether *mRAD54* is also involved in homologous recombination in MEFs, we performed gene targeting experiments with an isogenic *mRAD54-HisHA*$^{cDNA\text{-}307hyg}$ knockin targeting construct. The details of this construct are described in Figure 1 of Chapter 7. It contains a hygromycin selectable marker and only homologously targeted integration of this construct at the *mRAD54* locus produces a full-length HA-tagged mRad54 protein. Targeting efficiency was measured as the percentage of hygromycin-resistant clones that expressed mRad54-HA due to homologous integration of the targeting construct relative to the total number of hygromycin resistant clones that were analyzed (Table 3). In two separate experiments, the targeting efficiency decreased from 58% in the *mRAD54*-proficient MEF line to 4% in the *mRAD54*-deficient MEF line and from 50% to 28%, respectively. Mouse ES cells are seen as rare among mammalian cell types since targeted integration in ES cells can be the predominant event compared to illegitimate recombination. However, the key step of efficient targeted recombination is the use of isogenic DNA, which has been extensively investigated in ES cells [24], but rarely in other cell types [25, 26]. We conclude that disruption of *mRAD54* reduces homologous recombination not only in ES cells but also in MEFs.

Subsequently, we tested whether the sensitivity of *mRAD54*$^{-/-}$ ES cells to the crosslinking agent mitomycin C was also found in *mRAD54*$^{-/-}$ mice. We measured the effect of various doses of the drug, ranging from 7.5 to 15 mg/kg, on survival (Figure 3). Injection of 10 mg/kg and 7.5 mg/kg mitomycin C was lethal for 50% of *mRAD54*$^{-/-}$ females and males, respectively, and 0% of the wild-type mice (Figure 3A and 3C). Injection of 15 mg/kg and 10 mg/kg mitomycin C into females and

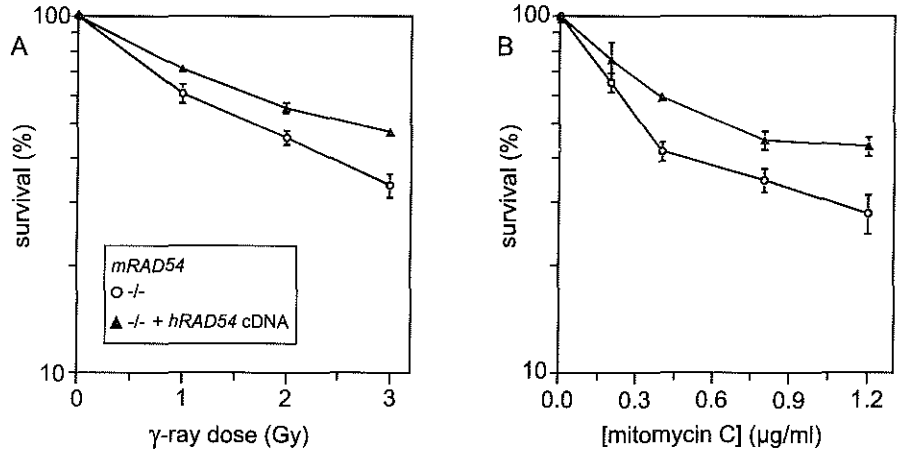

Figure 2. Effect of IR and mitomycin C on *mRAD54*-proficient and -deficient MEFs. **A,** Shown are survival-curves of *mRAD54*-proficient and -deficient MEF cell lines after treatment with increasing doses of IR as measured by colony forming ability. Experiments were performed in duplicate and for each genotype two independent cell lines were used. **B,** Shown are survival curves of the same cell lines shown in A after treatment with increasing doses of mitomycin C.

males, respectively, also showed an enhanced sensitivity and a shorter latency period in *mRAD54*$^{-/-}$ mice (Figure 3B and 3D). No difference in lethality was observed between *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ mice (data not shown). These results show that adult *mRAD54*$^{-/-}$ mice are mitomycin C sensitive.

Since the bone marrow is a major target for mitomycin C-inflicted damage *in vivo*, we tested whether mitomycin C treatment differentially affected cells in the blood of *mRAD54*$^{+/-}$ and in *mRAD54*$^{-/-}$ mice. To this end we used the peripheral blood micronucleus assay which provides a measure of chromosomal aberrations in polychromatic erythrocytes (PCEs). The induction of micronuclei following treatment of *mRAD54*$^{-/-}$ and *mRAD54*$^{+/-}$ mice with mitomycin C was determined. Two-months old mice were treated with a single dose of mitomycin C. This treatment resulted in dose-related increases in the frequency of micronuclei containing PCEs (MNPCEs) as can be seen in Figure 4. Initial levels (0 mg/kg) of MNPCEs were the same in *mRAD54*$^{-/-}$ and *mRAD54*$^{+/-}$ mice. Mitomycin C-induced levels of MNPCEs were significantly higher in *mRAD54*$^{-/-}$ mice compared to *mRAD54*$^{+/-}$ mice after treatment with 1.0, 2.5 and 5.0 mg/kg (Figure 4). These results are in agreement with the observed *in vivo* mitomycin C sensitivity of adult *mRAD54*$^{-/-}$ mice.

We analyzed the role of homologous recombination in mammals, using the *mRAD54*$^{-/-}$ mouse as the first viable mouse model with a defect in homologous recombination. Since we unexpectedly found that adult *mRAD54*$^{-/-}$ mice are not IR sensitive, we determined the IR sensitivity of early embryonic stages of *mRAD54*$^{-/-}$

Comparison of different phenotypes observed in ES cells, MEFs and mice: Fold reduction or sensitivity due to *mRAD54*-deficiency is indicated

	homologous recombination	IR	mitomycin C
ES cells	5-10	2.3	2-3
MEFs	2-10	1.4	2.0
Mice		1.0	1.5

Table 2. Disruption of *mRAD54* reduces homologous recombination in both ES cells and MEFs. *mRAD54*$^{-/-}$ ES cells are sensitive to IR and mitomycin C. The IR and mitomycin C sensitivity of *mRAD54*$^{-/-}$ MEFs is less pronounced. IR resistance in the adult mice is not affected by disruption of *mRAD54*, but adult *mRAD54*$^{-/-}$ mice are sensitive to mitomycin C. The quantitation of the observed sensitivities in ES cells and MEFs was obtained by comparing the slopes of the survival curves. The quantitation of the sensitivities in mice was derived from LD50 values for *mRAD54*$^{-/-}$ and *mRAD54*$^{+/+}$ mice.

mice. ES lines isolated *de novo* from *mRAD54*$^{-/-}$ embryos consisting of a mixed background of C57Bl6 and 129 DNA were IR sensitive compared to their *mRAD54*-proficient control ES cell lines. In addition, we found that *mRAD54*$^{-/-}$ day 3.5 embryos are IR sensitive compared to wild-type embryos. Similar radiation sensitivity assays with *mRAD51* and *BRCA1* mutant embryos also demonstrated their IR sensitivity [19, 20], reflecting an essential role of homologous recombination in IR resistance early in development. As the IR sensitivity of *mRAD54*$^{-/-}$ MEFs is less pronounced and *mRAD54*$^{-/-}$ mice are not IR sensitive (Table 2), we favor the hypothesis that *mRAD54*-mediated homologous recombination has an important role in conferring IR resistance early in development in less differentiated cell types. There are various explanations for the fact that although *mRAD54*$^{-/-}$ ES display a clear phenotype, the observed phenotype of adult *mRAD54*$^{-/-}$ mice is relatively mild. A possible explanation for the mild phenotypes of the adult *mRAD54*$^{-/-}$ mice observed to date, is redundancy of the mRad54 function. This redundancy could occur within the homologous recombination pathway itself or between the homologous recombination pathway and the DNA end-joining pathway.

An example of redundancy in Rad54 function within the homologous recombination pathway is found in *S. cerevisiae*. Recently, the *ScRDH54/TID1* gene was identified as having sequence similarity to *ScRAD54* [27, 28]. Although *ScRDH54/TID1* mutants show defects in meiotic cells and not in mitotic cells, the double *ScRAD54-ScRDH54/TID1* mutants show an increased methyl methanesulfonate sensitivity of mitotic cells compared to the single *ScRAD54* mutants. It is clear that *ScRAD54* and *hRAD54* are most closely related, but the relationship with the *ScRDH54/TID1* homolog is less clear. A potential second mammalian *ScRAD54* homolog has been identified and named *mRAD54B* (J.-M. Buerstedde, personal communication). This gene is clearly not as closely related to *ScRAD54* as *mRAD54* itself. However, because of the significant evolutionary

distance between both mammalian *RAD54* genes, it is not possible to unambiguously determine their relationship to *ScRAD54* and *ScRDH54/TID1*. Mice with homozygous disruptions in the *mRAD54B* gene are viable, fertile, and do not show gross abnormalities (J.-M. Buerstedde, personal communication). Double mutant mice carrying homozygous disruptions of *mRAD54* and *mRAD54B* have been generated (our unpublished results). They will provide valuable tools in analyzing potential redundancy between the Rad54-like proteins in mammals.

Homologous recombination is reduced in *mRAD54*$^{-/-}$ MEFs: Percentage of homologous integration events among total number of integration events

MEF genotype	Experiment 1	Experiment 2
mRAD54$^{+/-}$	58% (14/24)	50% (13/26)
mRAD54$^{-/-}$	4% (1/24)	28% (5/28)

Table 3. Disruption of *mRAD54* reduces homologous recombination in both ES cells and MEFs. *mRAD54*$^{-/-}$ ES cells are sensitive to IR and mitomycin C. The IR and mitomycin C sensitivity of *mRAD54*$^{-/-}$ MEFs is less pronounced. IR resistance in the adult mice is not affected by disruption of *mRAD54*, but adult *mRAD54*$^{-/-}$ mice are sensitive to mitomycin C. The quantitation of the observed sensitivities in ES cells and MEFs was obtained by comparing the slopes of the survival curves. The quantitation of the sensitivities in mice was derived from LD50 values for *mRAD54*$^{-/-}$ and *mRAD54*$^{+/+}$ mice

Besides redundancy within pathways, redundancy between pathways could possibly account for the mild phenotype of the adult *mRAD54*$^{-/-}$ mice. In this regard, it is interesting to compare the similarities and differences in phenotypic consequences of mutations in homologous recombination and DNA end-joining. The IR sensitivity of *mRAD54*$^{-/-}$ ES cells is quantitatively similar to that of mouse *Ku70*$^{-/-}$ ES cells [29]. Ku70 is one of the components of DNA-PK which is involved in DSB repair through DNA end-joining (Chapter 1). Thus, both DNA end-joining and homologous recombination can equally contribute to IR resistance in ES cells. However, adult mice defective in either *mRAD54*-dependent homologous recombination or DNA-PK-mediated DNA end-joining differ in IR sensitivity. We found that *mRAD54*$^{-/-}$ mice are not IR sensitive, while a mutation in DNA-PKcs or Ku80, two other components of DNA-PK, confer a 2- to 3-fold IR sensitivity to adult mice [30, 31]. A possible explanation is that accurate repair is crucial for mammalian stem cells and germ cells, whereas inaccurate repair could be more easily tolerated by differentiated somatic cells, given that a large fraction of their genome is no longer functional. Following this hypothesis, the DNA end-joining pathway could then confer IR resistance to the adult animal. However, in the absence of DNA end-joining, DNA repair through homologous recombination could

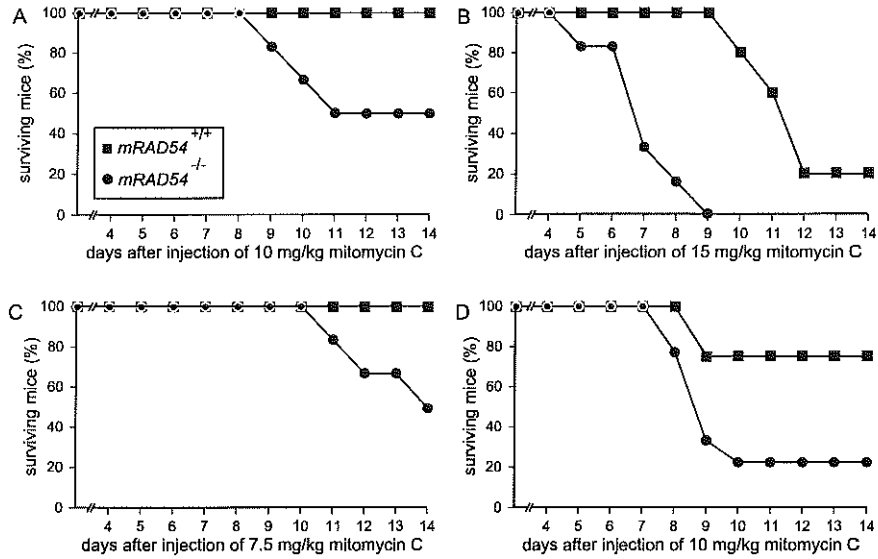

Figure 3. Mitomycin C sensitivity of *mRAD54*$^{-/-}$ mice. Shown are survival curves of *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice after a single intraperitoneal injection of the indicated amounts of mitomycin C. **A,** Survival curve of six *mRAD54*$^{+/-}$ female and six *mRAD54*$^{-/-}$ female mice after injection at day 0 with a dose of 10 mg/kg mitomycin C. **B,** Survival curve of five *mRAD54*$^{+/-}$ female and six *mRAD54*$^{-/-}$ female mice after injection with 15 mg/kg mitomycin C. **C,** Survival curve of eight *mRAD54*$^{+/-}$ male and nine *mRAD54*$^{-/-}$ male mice after injection with 7.5 mg/kg mitomycin C. **D,** Survival curve of six *mRAD54*$^{+/-}$ male and eight *mRAD54*$^{-/-}$ male mice after injection with 10 mg/kg mitomycin C.

become an important back-up repair mechanism. The reverse of this hypothesis has clearly been demonstrated in *S. cerevisiae*. The deletion of *S. cerevisiae* genes involved in DNA end-joining, such as *ScKu70, ScKu80* and *DNL/LIG4* does not cause sensitivity to IR. However, deletion of homologous recombination by deletion of *RAD52* together with the deletion of *ScKu70, ScKu80* or *DNL/LIG4* results in an increased IR sensitivity compared to the IR sensitivity of single *RAD52* mutants (see Chapter 1, Section 6). This hypothesis for mammals can be tested with the use of double mutant mice. Mice carrying the *scid* mutation and *mRAD54*-deficiency should have defects in both homologous recombination and DNA end-joining and could therefore reveal possible overlapping roles between the two DNA repair systems. In addition, the tissue specific, relative contribution to IR resistance of each of the two pathways can then be addressed by comparing the IR sensitivities of the different tissues of *scid*, *mRAD54*$^{-/-}$, and the *scid/mRAD54*$^{-/-}$ double mutant mice. Possible additional DSB repair capacity in *scid/mRAD54*$^{-/-}$ double mutant mice might be due to redundancy in gene function, but could also be due to an additional, yet unidentified, DSB repair system. One potential manner to distinguish between these two possibilities is to cripple both pathways even further.

Disruption of both *mRAD54* and *mRAD54B* probably reduce homologous recombination to a level below that found in *mRAD54*$^{-/-}$ cells, while the null mutant of *scid*, the DNA-PKcs$^{-/-}$ mouse, or null mutants of other subunits of DNA-PK, Ku70 and Ku80, would further cripple the DNA end-joining pathway. Viable triple knockouts of *RAD54*, *mRAD54B* and one of the subunits of DNA-PK, would then be the evidence for the existence of, physiological significant, additional DSB repair mechanisms.

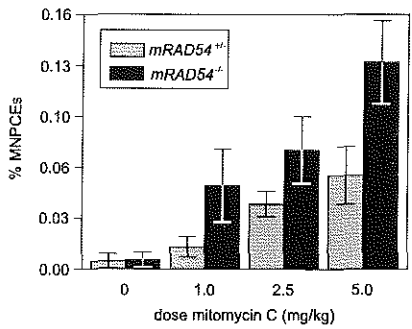

Figure 4. Induction of micronuclei by mitomycin C in polychromatic erythrocytes (PCEs). *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice were intraperitoneally injected with a single dose of 1.0, 2.5 and 5.0 mg/kg bodyweight mitomycin C. At 24 hours before and 48 hours after mitomycin C treatment, 25 µl of peripheral blood was collected by orbita punction. Per animal, 1000 PCEs were observed and the number of cells with micronuclei were recorded. Plotted are the number of micronuclei-containing PCEs (MNPCEs) per 1000 PCEs. Data points represent an average from four independently treated animals. The standard error of the mean is indicated for each measurement.

Interestingly, we found that *mRAD54*$^{-/-}$ ES cells and mice are sensitive to the crosslinking agent mitomycin C [2], while cells or mice containing the *scid* mutation are not mitomycin C sensitive [30]. DSBs are likely intermediates in the repair of these interstrand DNA crosslinks. In theory, both homologous recombination and DNA end-joining should be capable of repairing these DSBs. Given the DNA damage specificity we observed of *mRAD54*$^{-/-}$ and *scid* mice, it appears that the repair of DNA crosslinks is a specialized function of the homologous recombination pathway. We propose two possible explanations for this observation. Repair of a mitomycin C-induced DNA crosslink presumably requires two DSBs around the lesion. Removal of the crosslink by DNA end-joining would require that the two DSBs are made more or less simultaneously. If they are not made simultaneously, then the DNA at the undamaged end of the DSB would be joined to the DNA that still contained the crosslink and the repair cycle would be futile. In contrast, homologous recombination is very well capable of by passing a crosslink starting from a single DSB. Alternatively, the use of homologous recombination instead of DNA end-joining for the repair of DNA interstrand crosslinks could involve processing of the crosslink. It is possible that the crosslinked region undergoes extensive nuclease digestion. This would result in large ssDNA regions. These large ssDNA regions cannot be joined by DNA end-joining and most likely require homologous recombination for their repair. Double mutant mice containing mutations in both homologous recombination and DNA end-joining can possibly reveal such specialized roles in crosslink repair for either of the DSB repair mechanisms.

METHODS

***RAD54* cDNA rescue constructs.** A cDNA construct encoding hRad54 containing an amino-terminal poly-histidine tag and a carboxy-terminal hemagglutin tag was used to rescue the phenotype of the *mRAD54*$^{-/-}$ cells [23]. For expression in MEFs the cDNA was placed under control of the SV40 promoter (*SV40-hRAD54*). For expression in mouse ES cells the cDNA was placed under control of the PGK promoter (*PGK-hRAD54*).

Cell culture. ES cells, isolated from a 129 mouse strain, were cultured and electroporated as described [32]. To isolate *de novo* C57Bl6/129 ES cells *mRAD54*$^{-/-}$ embryos were isolated 3.5 days after a C57Bl6/129 *mRAD54*$^{-/-}$ cross. These *mRAD54*$^{-/-}$ mice were littermates from a *mRAD54*$^{+/-}$ cross, obtained by backcross three of an *mRAD54* chimera to C57Bl6 mice. Day 3.5 embryos were isolated and individually placed into a well of a 24-well plate. After incubation for approximately seven days without medium change, single inner cell mass outgrowths were carefully dislodged and taken up with as little medium as possible. After incubation for 5 min at 37°C in 100 µl trypsin/EDTA, they were mechanically dissociated into small cell clumps (4 to 6 cells). Finally, they were transferred to a gelatinized well of a 24-well plate. Medium was changed every other day and colonies with ES cell-like morphology were picked 7 to 14 days after plating the dissociated inner cell masses. Colonies were enzymatically and mechanically dissociated, and plated as described above for inner cell mass outgrowths. From then on the *de novo* isolated C57Bl6/129 ES cells were cultured as 129 ES cells. The *PGK-hRAD54* construct described above was electroporated into C57Bl6/129 *mRAD54*$^{-/-}$ ES line *mRAD54*$^{307neo/neo}$. Both the disrupted *mRAD54* alleles in this line contain the neomycin selectable marker. The *PGK-hRAD54* construct was co-electroporated with a plasmid carrying the puromycin selectable marker. Clones were selected as described [2] and screened for hRad54 expression by immuno-blot analysis.

After mating *mRAD54*$^{+/-}$ mice, MEFs were isolated from day 12-13 embryos. Part of the embryo was used for genotyping. The remaining embryonic tissue was minced using a pair of scissors and immersed in a thin layer of F10/DMEM culture medium supplemented with 10% FCS and antibiotics. Spontaneously immortalized cell lines were obtained by continuous subculturing of primary MEFs. To obtain *mRAD54*-proficient cell lines of an immortalized *mRAD54*$^{-/-}$ MEF cell line, the *SV40-hRAD54* construct was co-transfected, using lipofectin (Gibco), with a plasmid carrying the puromycin selectable marker. Clones were selected with puromycin (1 µg/ml) for 14 days. Integration of the cDNA construct and hRAD54 expression was confirmed by DNA- and immuno-blot analysis, respectively. Two clones that did not express hRad54 (#32 and #33) and two hRad54-expressing clones (#10 and #36) derived from this transfection were used for cell survival analyses (see below).

Cell survival assays. The sensitivity of ES cells to increasing doses of IR was determined by measuring their colony forming ability after irradiation with a ^{137}Cs source. Cells were grown for 7 to 10 days, fixed, stained, and counted. All measurements were performed in triplicate. The sensitivity of MEFs to increasing doses of DNA-damaging agents was also determined by measuring their colony forming ability. Various dilutions of the *mRAD54*-deficient and -proficient MEFs were placed into 10 mm dishes and after 12 to 24 hrs, cells were incubated for 1 hr in mitomycin C-containing media. IR sensitivity was determined by comparing the colony-forming ability of the MEFs after irradiation with a ^{137}Cs source. Cells were grown for 7 to 10 days, fixed, stained, and counted. All measurements were performed in duplicate.

Embryo culture and radiation treatment. *mRAD54*$^{+/+}$ embryos were isolated 3.5 days after a C57Bl6/129 mating. Similarly, embryos were isolated from a C57Bl6/129 *mRAD54*$^{-/-}$ mating. All animals were littermates from a *mRAD54*$^{+/-}$ cross, obtained from backcross three of a *mRAD54* chimera (129) to C57Bl6 mice. Immediately after isolation, embryos were exposed to IR from a ^{137}Cs source, plated in gelatinized wells with a diameter of 1 cm and cultured in ES cell medium. Outgrowth of the trophoblast-like cells and/or the inner cell mass was determined on day 10.

***In vivo* IR and mitomycin C survival.** For *in vivo* IR survival experiments, 2- to 4- months old animals of each genotype were exposed to IR from a ^{137}Cs source. Single doses between 6 and 8 Gy were used. For *in vivo* mitomycin C survival, 2-months old *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ animals were intraperitoneally injected with 7.5, 10 or 15 mg/kg bodyweight mitomycin C. After exposure to IR or injection with mitomycin C, mice were kept in sterile isolators and observed for 28 and 14 days, respectively. After these periods surviving animals were euthanized. All animal experiments were approved by the animal welfare committee of the National Institute of Public Health and Environmental Protection.

Micronucleus assay after *in vivo* mitomycin C treatment. Two-months old *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ animals were injected intraperitoneally with 1.0, 2.5 or 5.0 mg/kg bodyweight mitomycin C. At 24 hours before and 48 hours after mitomycin C treatment 25 µl of peripheral blood was collected by orbita punction. The blood was placed on an acridine orange coated glass slide, covered with a coverslip, and allowed to stain [33]. Erythrocytes with a red fluorescing reticulum in the cytoplasm were observed. 1000 erythrocytes were observed per animal by fluorescence microscopy within a few days of slide preparation. The number of cells with micronuclei displaying greenish yellow fluorescence were recorded.

Homologous recombination in mouse embryonic fibroblasts. An approximately 9-kb genomic *mRAD54* *Eco*RI fragment encompassing exons 4, 5, and 6 and

containing a unique *Sfu*I site in exon 4 was subcloned in pBluescript II KS (Stratagene). The 5'-end of the fusion construct contained the genomic DNA up to the *Sfu*I site in exon 4 corresponding to amino acid position 307 in the mRad54 protein. The 3'-end of the construct was derived from the *mRAD54* cDNA starting at the *Sfu*I site in exon 4. The cDNA had been modified to encode a carboxy-terminal poly-histidine and hemagglutin tag which were followed by a p(A) signal sequence and a PGK-promoter driven hygromycin expression cassette. The construct is schematically depicted in Figure 1 of Chapter 7. The resulting targeting vector is referred to as *mRAD54-HisHA*$^{cDNA\text{-}307hyg}$. Routinely, $1x10^7$ spontaneously immortalized MEFs were transfected with 10 μg linearized *mRAD54-HisHA*$^{cDNA\text{-}307hyg}$, using lipofectin (Gibco). Clones were selected with hygromycin (500 μg/ml) for 14 days. Targeted integration of the *mRAD54-HisHA*$^{cDNA\text{-}307hyg}$ construct was assayed by determining mRad54-HisHA expression by immuno-blot analysis, using a monoclonal α-HA antibody.

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CHAPTER 6

The human Rad54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase

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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).

mRAD54$^{\Delta 07neo/\Delta pur}$ (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, 4.5×10^8 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 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A, and 1 μg/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 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A, and 1 μg/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 β-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 β-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 β-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 4.5×10^8 infected Sf21 cells varied between 60 and 120 μg 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 μg/ml bovine serum albumin, 200 μM ATP, 0.25 μCi of [γ-^{32}P]ATP (>5000 Ci/mmol), 45 μM DNA (concentration in nucleotides) and 0–100 ng of hRad54 or hRad^{K189R} protein in a 10-μl 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 [γ-^{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 *XmaI-BglII* restriction fragment from the *hRAD54* cDNA (21) and treating it with Klenow DNA polymerase in the presence of dTTP, dGTP, dCTP, and [α-^{32}P]dATP. This procedure resulted in incorporation of radiolabel in one of the two strands, indicated by the underlined nucleotide in italics; 5′-CCGGGTCTGGCGAGATGGTCAAAAGAAGACTTGCTATATCTACCGCCTGCTGTCTGCAGGGACCATTGAGGAGAAGATC.

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 [γ-^{32}P]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 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

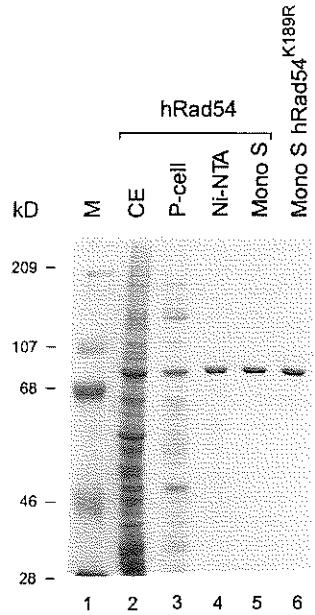

FIG. 1. **Purification of the hRad54 protein.** Coomassie-stained SDS-polyacrylamide gel containing samples taken at different stages of the hRad54 purification. A protein extract from Sf21 cells infected with a baculovirus expressing hRad54 (*CE*) (*lane 2*) was fractionated sequentially over phosphocellulose (*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 μg 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).

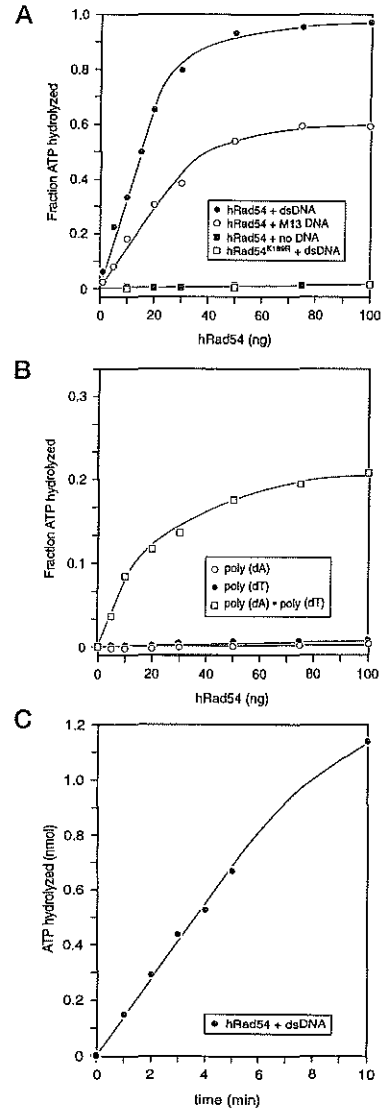

FIG. 2. **The hRad54 protein is a dsDNA-dependent ATPase.** *A*, the amount of ATP hydrolyzed by hRad54 and $hRad54^{K189R}$ after 60 min in the presence and absence of DNA is displayed as a function of enzyme concentration. The amount of ATP in the reaction mixture at time 0 was 2 nmol. ATPase assays were carried out as described under "Experimental Procedures." Reaction mixtures (10 μl) 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

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,

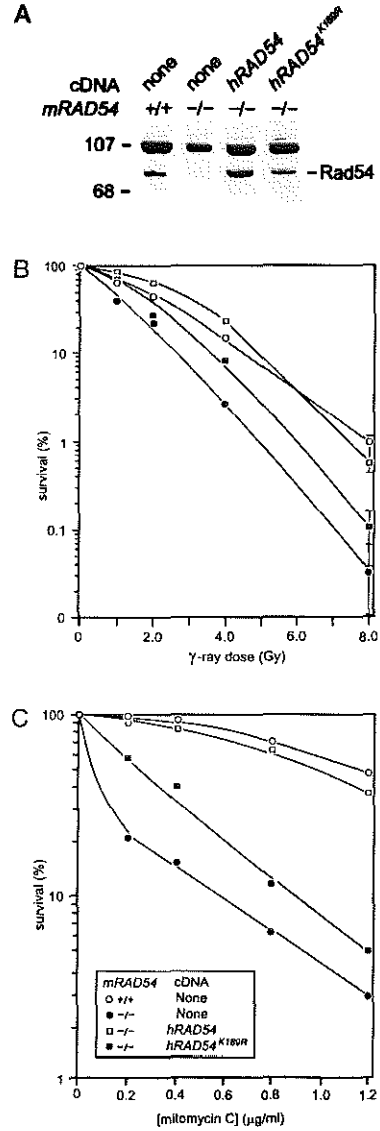

FIG. 3. **hRad54 rescues the γ-ray and mitomycin C sensitivity of *mRAD54* knockout ES cells.** *A*, immunoblot of protein extracts from wild-type (+/+) and *mRAD54* knockout (-/-) ES cells. *mRAD54* knockout ES cells were stably transfected with the wild-type (*hRAD54*) or mutant (*hRAD54*^{K189R}) 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.

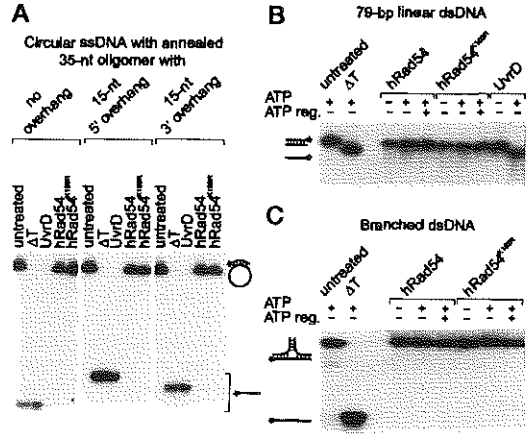

FIG. 4. **The hRad54 protein displays no activity in DNA helicase assays.** *A*, DNA helicase assay using a 35-nucleotide (*nt*) oligomer annealed to M13 viral DNA as a substrate. The three oligonucleotides used were either completely complementary or contained a 15-nucleotide 5′ or 3′ noncomplementary 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 (*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 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

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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CHAPTER 7

The mouse Rad54 recombinational DNA repair protein alters DNA structure and is required for formation of DNA damage-induced Rad51 foci

Submitted for publication

THE MOUSE Rad54 RECOMBINATIONAL DNA REPAIR PROTEIN ALTERS DNA STRUCTURE AND IS REQUIRED FOR FORMATION OF DNA DAMAGE-INDUCED Rad51 FOCI

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Error-free repair of ionizing radiation (IR)-induced DNA double-strand breaks (DSBs) by homologous recombination requires the Rad51 and Rad54 proteins in the yeast *Saccharomyces cerevisiae*[1-3]. Biochemical experiments have shown that the central events of recombination, homologous DNA pairing and strand exchange are mediated by Rad51 and that Rad54 can stimulate the recombination activities of Rad51 (refs 4,5). Mammalian homologs of Rad51 and Rad54 have been identified[3,6-9]. Mouse embryonic stem (ES) cells lacking Rad54 are IR sensitive and show reduced levels of homologous recombination[9]. Here we demonstrate that mouse Rad54 forms IR-induced nuclear foci that colocalize with Rad51. Interaction between mouse Rad51 and Rad54 is induced by genotoxic stress, but only due to lesions whose repair requires Rad54. Interestingly, mouse Rad54 is essential for the formation of IR-induced Rad51 foci. Rad54 belongs to the SWI2/SNF2 protein family whose members modulate protein-DNA interactions in an ATP-driven manner[10]. We show that in the presence of a ligase, purified human Rad54 protein introduces negative supercoils in nicked DNA at the expense of ATP hydrolysis. The ability of Rad54 to alter DNA structure and its requirement for genotoxic stress-induced Rad51 foci formation suggest a key role for Rad54 in recombination.

To study the behavior of mouse Rad54 protein (mRad54) after IR treatment of cells, a knockin construct was generated in which exons 4-18 were replaced by the corresponding cDNA encoding a carboxy-terminal hemagglutin (HA) tag. Homologous integration of the construct within the *mRAD54* locus of ES cells ensured expression of HA-tagged mRad54 from the endogenous *mRAD54* promoter (Figure 1A). The protein (88 kDa) was detected using α-hRad54 and α-HA antibodies on immunoblots from cell lines *mRAD54*$^{-/HA}$ and *mRAD54*$^{+/HA}$. These cells contain knockout and wild-type *mRAD54* alleles, respectively, in addition to the HA-tagged allele (Figure 1B). The HA-tag did not interfere with the biological activity of mRad54 since cells expressing only the tagged protein displayed the same IR resistance as cells expressing untagged mRad54 (Figure 1C).

*These authors contributed equally to the work

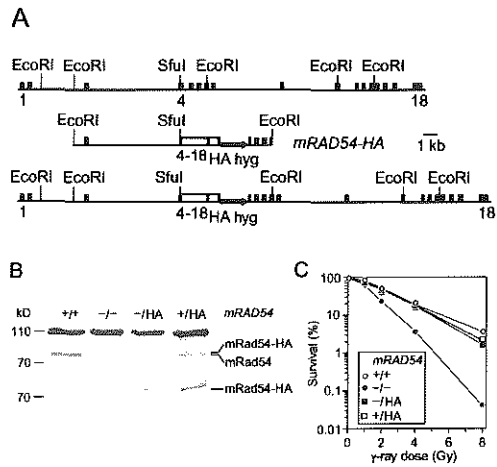

Figure 1. Generation and characterization of ES cell lines containing endogenously expressed HA-tagged mRad54. **A**, Structure of the genomic *mRAD54* locus (top), the HA-tagged *mRAD54* knockin construct (middle), and the targeted locus (bottom). Black boxes represent the 18 exons encoding mRad54. HA, hemagglutin tag; hyg, hygromycin resistance gene. **B**, Immunoblot of extracts from *mRAD54* wild-type (+/+), knockout (-/-), and knockin (-/HA and +/HA) ES cells, probed with α-hRad54 and α-HA antibodies (upper and lower panel, respectively). The nature of the 110-kDa protein that cross-reacts with the α-hRad54 antibodies is unknown. **C**, Clonogenic survival assays of the indicated ES cell lines after IR treatment.

When the steady-state level of mRad54 was measured after treatment of cell line *mRAD54*$^{-/HA}$ with IR, no dose- or time-dependent difference in mRad54 levels was detected (Figure 2A). A similar result was obtained for mRad51. In contrast, an IR-induced increase in p53, a protein known to be induced upon IR treatment[11], was observed (data not shown). However, although the steady-state level of mRad54 did not change, the protein was redistributed after IR treatment, as detected by immunofluorescence microscopy (Figure 2B). After IR treatment, mRad54 was detected as bright foci in the nuclei of the majority of the cells. Since this staining pattern is similar to that reported for Rad51 (refs 12-14), cells were also stained for mRad51, revealing that the mRad54 and mRad51 foci colocalized (Figure 2B). DAPI staining of irradiated cells showed that foci-containing cells did not show signs of apoptosis or were otherwise aberrant.

To determine whether the IR-induced mRad51 and mRad54 foci colocalized because of an association of the two proteins, immunoprecipitation experiments were performed. Immobilized α-hRad51 antibodies were used to precipitate mRad51 from *mRAD54*$^{-/HA}$ ES cell protein extracts, and the precipitate was analyzed for the presence of mRad51 and mRad54. While mRad51 was detected in the precipitate from extracts of unirradiated and irradiated cells, mRad54 was co-immunoprecipated only from the extract of irradiated cells (Figure 2C). Similarly, IR-dependent co-immunoprecipitation of mRad51 and mRad54 was observed using immobilized α-HA antibodies (data not shown).

IR-induced mRad51 and mRad54 foci formation was examined in time to determine whether the redistribution of these proteins upon induction of genotoxic stress was a dynamic process. The percentage of cells showing both mRad51 and mRad54 foci, *i.e.* double positive cells, increased from 8.5 to 76% over a two hour period after IR treatment with doses of 2 to 12 Gy (Figure 3A). In contrast, the percentage of cells showing mRad51 or mRad54 foci only, did not change in a time-

or dose-dependent manner and did not differ from that found in untreated cells (data not shown). Nor was there any significant dose-dependent difference in the induction rate of mRad51 and mRad54 foci. However, the decrease in the percentage of double positive cells over time was dependent on IR dose. One day after treatment with 2 Gy, the percentage of double positive cells was reduced to levels found in untreated cells, while treatment with the lethal dose of 12 Gy did not result in a substantial reduction (Figure 3A).

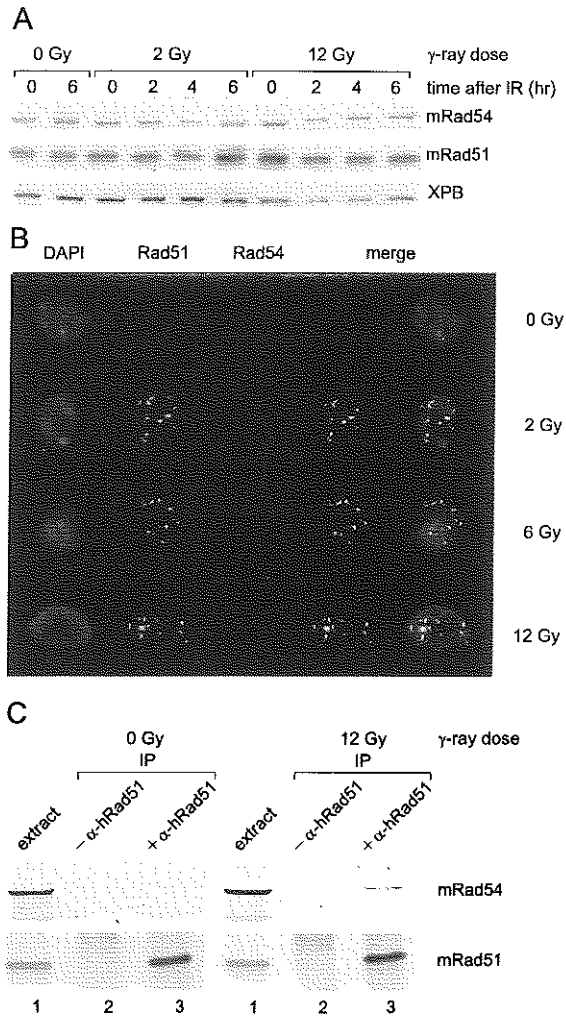

Figure 2. IR-induced interaction between mRad51 and mRad54. **A**, Immunoblots of protein extracts from ES cells (*mRAD54*$^{/HA}$) isolated at the indicated times after IR treatment and probed with antibodies against the indicated proteins. XPB served as a loading control. **B**, IR-induced colocalization of mRad51 and mRad54 foci as detected by immunofluorescence within cells fixed 6 hours after treatment. The first three columns show separate nuclear (DAPI), mRad51 and mRad54 staining, respectively, while the latter two show merged combinations (see back cover for full color image). **C**, Co-immunoprecipitation of mRad54 with immobilized α-hRad51 antibodies requires prior IR treatment of the cells. Lane 1, protein extracts; lane 2, immunoprecipitation (IP) using pre-immune serum; lane 3, IP using α-hRad51 antibodies. The immunoblots in the upper and lower panels were probed α-HA and α-hRad51 antibodies, respectively.

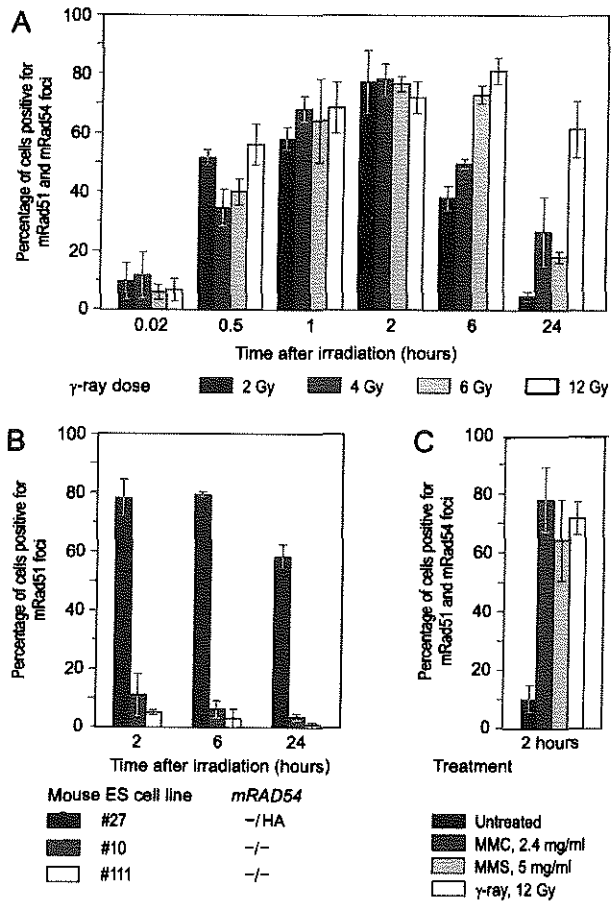

Figure 3. Kinetics of mRad51 and mRad54 foci formation. **A**, ES cells (*mRAD54*$^{/HA}$) were treated with the indicated doses of IR, fixed at given time points, and stained for mRad51 and mRad54. The percentage of cells containing both mRad51 and mRad54 foci is displayed. **B**, As in A except *mRAD54*$^{-/-}$ cells were used and the percentage of cells containing mRad51 foci is plotted. **C**, As in A, except that the cells were fixed two hours after treatment with the indicated agents.

To test whether the IR-induced interaction between mRad54 and mRad51 affects mRad51 foci formation, mRad51 foci formation was examined in *mRAD54*$^{-/-}$ ES cells. Interestingly, IR did not induce mRad51 foci formation in *mRAD54*$^{-/-}$ cells (Figure 3B). This effect was observed in two independently obtained *mRAD54*$^{-/-}$ cell lines. Importantly, the *mRAD54*-proficient and deficient cell lines were isogenic and differed solely in their *mRAD54* alleles. We conclude that mRad54 is required for DNA damage-induced mRad51 foci formation. Recently, the Rad51 family member XRCC3 was also shown to be required for Rad51 foci formation[15].

To determine whether other types of DNA damage elicit mRad54 foci formation, cells were treated with mitomycin C (MMC), methyl methanesulfonate (MMS) and UV-light. Foci containing both mRad51 and mRad54 were induced by

MMC and MMS, but not by UV-light (Figure 3C), which correlates with the DNA damage sensitivities of *mRAD54*$^{-/-}$ ES cells[9]. This result is consistent with the observation that MMS treatment induces Rad51 foci in both primary and transformed mammalian cells[12,13]. We conclude that the mRad54 protein redistributes in the cell and associates with mRad51 upon induction of genotoxic stress generated by DNA damage that requires mRad54 for its repair. In contrast, no redistribution takes place in response to lesions whose repair does not depend on mRad54. Our observations are consistent with experiments showing that Rad51 and Rad54 from *S. cerevisiae* and human can interact *in vitro*[16-18]. However, in contrast to the *S. cerevisiae* proteins, which interact under normal physiological conditions, significant interaction between the mouse proteins requires DNA damage.

In addition to the physical interaction, Rad51 and Rad54 interact functionally. During recombinational repair, Rad51 mediates joint molecule formation between a single-stranded region on the damaged DNA and an intact homologous duplex DNA. Rad51 initiates homologous pairing by forming a nucleoprotein filament on the single-stranded DNA[19]. The *S. cerevisiae* Rad54 protein has been shown to stimulate the pairing activity of Rad51[5]. However, the molecular basis of this stimulation is not understood. *S. cerevisiae* and human Rad54 (hRad54) are double-stranded DNA-dependent ATPases that belong to the SNF2/SWI2 protein family[5,20]. Although members of this family contain seven conserved motifs characteristic of helicases[10,21], helicase activity of these proteins using oligonucleotide displacements assays has not been detected[5,10,20]. One interpretation of these results is that although proteins of this family do not disrupt base pairing, they might still be able to locally unwind the DNA double helix. To further investigate this possibility the interaction of hRad54 with double-stranded DNA was examined using a topological assay. Singly-nicked plasmid DNA was incubated with hRad54 and the reaction mixture was then supplemented with DNA ligase. Any protein-induced change in linking number (ΔLk) will be detected as a change in the electrophoretic mobility of the DNA. In the presence of ATP, hRad54 generated topoisomers that migrated with native superhelix density DNA (Figure 4A), indicating that the protein induced an extensive ΔLk. The amount of converted DNA increased with increasing hRad54 concentration (Figure 4B). The hRad54-induced ΔLk required ATP hydrolysis since it was not observed in the absence of ATP, in the presence of the non-hydrolyzable analog ATPγS (Figure 4B), or with hRad54^{K189R} which carries a single amino acid substitution that blocks ATP hydrolysis (Figure 4A)[20].

The direction and extent of the hRad54-induced ΔLk was determined by two-dimensional gel electrophoresis. In the presence of ATP, hRad54 introduced negative supercoils in the plasmid DNA (Figure 4C). Topoisomers with a ΔLk of up to -23 were resolved, indicating that hRad54 binding can induce a specific linking difference ($\sigma = \Delta Lk/Lk_0$) of at least –0.08 which is even lower than that of native superhelix density DNA ($\sigma \cong -0.06$). Although a widening of the topoisomer distribution was observed in the absence of ATP, the centre of the distribution was unchanged. The negative supercoils introduced by hRad54 could either result from

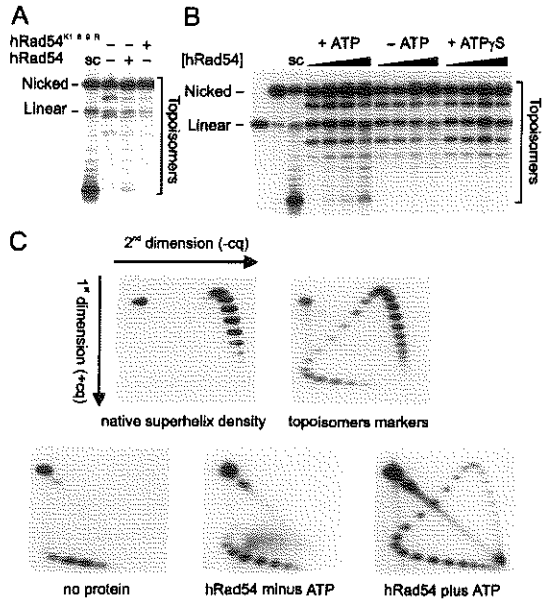

Figure 4. Binding of hRad54 to DNA induces negative supercoiling. **A**, Equal amounts of hRad54 and hRad54^{K189R} proteins were incubated with singly-nicked plasmid DNA in the presence of ATP. After ligation of the nick the resulting distribution of topoisomers was analyzed by agarose gel electrophoresis. sc, native superhelix density DNA. **B**, The induction of an extensive ΔLk by hRad54 is dependent on protein concentration and requires ATP hydrolysis. The amount of hRad54 used was 0, 60, 120, and 180 ng. **C**, Binding of hRad54 induces a negative ΔLk. Series of two-dimensional gels containing the indicated DNAs. cq, chloroquine.

a change in twist due to unwinding of the DNA double helix, or from a change in writhe due to DNA wrapping around the protein surface. Given that the *E. coli* recombination protein RuvB which contains similar conserved motifs to those found in Rad54 and the SNF2/SWI2-containing protein complex change twist[21-23], we favor the possibility that the negative supercoils induced by hRad54 are due to DNA unwinding.

We suggest that the stimulation of Rad51-mediated homologous DNA pairing by Rad54 is due to unwinding of the double-stranded DNA recombination partner[5]. Unwinding will facilitate pairing because the DNA in the Rad51 nucleoprotein filament is in an extended conformation[19]. In comparison with *E. coli* RecA protein, hRad51 makes only short heteroduplex joints[19]. Through its association with Rad51 and its ATP-dependent DNA unwinding activity hRad54 might provide stability to the hRad51-mediated joint allowing extension of the heteroduplex DNA. This role is consistent with our demonstration that hRad54 is required for the formation of Rad51 foci induced by genotoxic stress. In addition to a direct role in stimulating Rad51-promoted homologous pairing, Rad54 could influence pairing indirectly by affecting chromatin structure. Rad54-mediated DNA unwinding might result in displacement of histones that could be inhibitory to homologous pairing. Such a role is in agreement with the functions of other SNF2/SWI2 family members that have been implicated in chromatin remodeling and removal of proteins from DNA[10].

METHODS

Cell culture and survivals. ES cell culture and cell survival assays were carried out as described[9]. Measurements were performed in triplicate. Standard errors of the mean were within 4-16%, except for the dose of 8 Gy for the *mRAD54*$^{-/-}$ line which showed an error of 28%.

Immunoprecipitation. For immunopreciptiations, cells were lysed by resuspension in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, and 0.5 % Triton X-100 (NETT buffer). All manipulations were carried out at 4° C. The extract was clarified by centrifugation followed by the sequential addition of α-hRad51 antibodies and protein G sepharose beads to the supernatant. The beads were washed three times with NETT buffer. The immunoprecititate was fractionated by SDS-PAGE and gels were analyzed by immunoblotting using rabbit α-hRad51 and rat α-HA antibodies.

Immunofluorescence. Cells were grown on gelatinized glass slides, treated with DNA damaging agents[9], fixed at different time points with methanol/acetone and processed as described[14]. Slides were incubated for 1.5 hours at 20° C with α-hRad51 and α-HA antibodies, followed by a 1.5 hours incubation with Alexa 488-conjugated goat α-rabbit and Alexa 594-conjugated goat α-rat secondary antibodies obtained from Molecular Probes. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Cells with two or more nuclear foci of one type were considered as positive for that type. Each data point, obtained in three independent experiments, was based on the analysis of at least 100 nuclei. Standard deviations are indicated in Figure 3. Image acquisition and processing were done as described[14].

Topological analysis. The purification of hRad54 is described elsewhere[20]. Singly-nicked plasmid DNA (pBluescript II KS) was prepared as described[24]. Reaction mixtures (60 µl) contained 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 26 µM NAD, 50 µg/ml BSA, and 50 ng DNA unless stated otherwise. After 10 min at 30° C, one unit *E. coli* DNA ligase was added and incubation was continued for 50 min. Purification of DNA and two-dimensional gel electrophoresis were performed as described[24,25]. Marker topoisomers were generated by topoisomerase I in the presence of different concentrations of ethidium bromide.

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SUMMARY

DNA double-strand breaks (DSBs) are major threats to the integrity of DNA inside cells, as they interfere with key steps in DNA metabolism such as replication and transcription. A broad range of factors, including endogenously produced radicals and ionizing radiation (IR) generates DSBs. DSBs are also possible intermediates during repair of interstrand DNA crosslinks, which can be introduced by crosslinking agents such as mitomycin C. The accurate repair of DSBs is essential to prevent chromosomal fragmentation, translocations, and deletions. The persistence of these chromosomal aberrations can lead to carcinogenesis through activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity. Insight into the mechanism of DSB repair in mammals is crucial in order to understand the biological consequences of exposure to IR. Beside the importance of DSB repair for the maintenance of genomic integrity, the extensive use of IR in anti-cancer therapy necessitates a broadening of our knowledge into the mechanism of DSB repair in mammals.

In **Chapter 1**, the role and molecular mechanism of the multiple pathways that have evolved for the repair of DSBs are discussed. The existence of multiple pathways is probably a reflection of the extreme genotoxicity of DSBs. Two major DSB repair pathways are homologous recombination and DNA end-joining. Homologous recombination requires extensive regions of DNA homology and repairs DSBs accurately using the information on the undamaged sister chromatid or homologous chromosome. DNA end-joining uses no or extremely limited sequence homology to rejoin broken ends in a manner that need not be error-free. Thus, a critical difference between these two mechanisms is the fidelity of repair. While homologous recombination ensures accurate DSB repair, DNA end-joining does not. The initial genetic analysis of IR sensitive rodent cell lines has been crucial in identifying genes involved in DSB repair. Interestingly, the isolated mammalian genes were found to be involved in DSB repair through DNA end-joining, and not through homologous recombination. However, recent studies reveal the functional conservation of the protein machinery involved in homologous recombination from fungi to humans, implying an important function for the mammalian homologous recombination genes.

Chapters 2 to 7 describe the generation and phenotypic characterization of cells and mice, with a defect in the *RAD54* recombinational DNA repair gene. Furthermore, the initial characterization and cellular behavior of the mammalian Rad54 protein is described. One of the key genes in homologous recombination in the yeast *Saccharomyces cerevisiae* is *RAD54* (*ScRAD54*). Based on its primary amino acid sequence, ScRad54 belongs to the SNF2/SWI2 protein family. The SNF2/SWI2 protein of the yeast *S. cerevisiae* is part of an evolutionary conserved multiprotein complex involved in chromatin remodeling. Members of the SNF2/SWI2 family of proteins have been implicated in cellular processes such as

transcription, repair, and recombination, illustrating that the individual members of this family have distinct functions.

In **Chapter 2**, the identification of the human (*hRAD54*) and mouse (*mRAD54*) homologs of the *ScRAD54* gene is described. At the primary amino acid sequence level, the hRad54 and mRad54 proteins are 48% identical to the ScRad54 protein. The *hRAD54* gene is located on human chromosome 1p32. By complementation analysis with a cDNA construct expressing hRad54 in *ScRAD54* mutant cells, we showed that the DNA repair function of Rad54 is conserved from yeast to humans.

In **Chapter 3**, the isolation and characterization of the *mRAD54* locus are described. We determined the *mRAD54* genomic organization to generate gene targeting constructs for the disruption of *mRAD54* in cells and mice. *In situ* hybridization experiments showed that the *mRAD54* gene resides on mouse chromosome 4, near band C7/D1, a region syntenic to human chromosome 1p32.

In **Chapter 4**, the generation of *mRAD54*$^{-/-}$ embryonic stem (ES) cells and *mRAD54*$^{-/-}$ mice is described. To examine the role of mRad54 in ES cells, we determined the sensitivities towards different DNA damaging agents and the efficiency of homologous recombination in *mRAD54*$^{-/-}$ ES cells. Since *ScRAD54* mutants are sensitive to IR, we tested the IR-sensitivity of *mRAD54*$^{-/-}$ ES cells. *mRAD54*$^{-/-}$ ES cells are approximately 2-fold more sensitive to IR than wild-type ES cells. With the use of isogenic targeting constructs for the *RB* and *CSB* loci, we showed that *mRAD54* is required for efficient homologous integration in ES cells. This result links a decrease in homologous recombination to IR sensitivity in mammalian cells. To determine the biological effect of deleting *mRAD54* at the whole animal level, we generated *mRAD54*$^{-/-}$ mice. The inheritance of the mutated *mRAD54* allele is Mendelian and *mRAD54*$^{-/-}$ mice are apparently healthy without any gross abnormalities.

In **Chapter 5,** the characterization of the *mRAD54*$^{-/-}$ mice is described. We unexpectedly found that adult *mRAD54*$^{-/-}$ mice are not IR-sensitive. However, embryonic stages of these *mRAD54*$^{-/-}$ mice, represented by *de novo* isolated *mRAD54*$^{-/-}$ ES cells and *mRAD54*$^{-/-}$ day 3.5 embryos did show enhanced IR-sensitivity compared to wild-type controls. To determine the effect of IR in a differentiated cell type, we isolated *mRAD54*$^{-/-}$ mouse embryonic fibroblasts (MEFs). *mRAD54*$^{-/-}$ MEFs were 1.4-fold more sensitive to IR and 2-fold more sensitive to the crosslinking agent mitomycin C, compared to their mRad54-proficient controls. To determine whether the process of homologous recombination was affected in *mRAD54*$^{-/-}$ MEFs, we determined the targeting efficiency at the *mRAD54* locus and found that homologous recombination was reduced in *mRAD54*$^{-/-}$ MEFs. The physiological significance and the protective role of mRad54 in the adult animal were assayed by the peripheral blood micronucleus assay and survival analysis after intraperitoneal injection of mitomycin C. Although *mRAD54*$^{-/-}$ mice are not IR sensitive, *mRAD54*$^{-/-}$ mice are sensitive to mitomycin C and show elevated levels of micronuclei induction after mitomycin C treatment, compared to control mice. In conclusion, mRad54-dependent homologous

recombination contributes to both IR and mitomycin C resistance early in development and only to mitomycin C resistance in the adult mouse. As DNA end-joining specifically contributes to IR resistance but not to mitomycin C resistance, this indicates that DNA end-joining and homologous recombination have overlapping roles in the repair of IR induced damage. However, repair of DNA interstrand crosslinks is dependent on homologous recombination.

In **Chapter 6**, the initial characterization of the hRad54 protein is described. In addition to our studies on the *mRAD54*$^{-/-}$ phenotype at the cellular and animal level, we also used a biochemical approach to study the function of the mammalian Rad54 protein. To facilitate protein purification, we tagged the hRad54 protein with an amino-terminal histidine and a carboxy-terminal hemagglutin-tag (HA-tag). Next, we showed that the addition of the tags did not interfere with the biological function of hRad54 because the tagged protein could rescue the IR and mitomycin C sensitivity of *mRAD54*$^{-/-}$ ES cells to wild-type levels. Introduction of a *hRAD54* construct encoding a protein containing a single amino acid substitution at position 189 (referred to as hRad54^{K189R}) led to a partial correction for both IR and mitomycin C sensitivity. Conversion of the lysine residue into an arginine at position 189 impairs ATP hydrolysis, without altering the overall structure of the hRad54 protein. The same mutation in the equivalent Walker box in a number of other ATPases severely impairs nucleotide triphosphate hydrolysis. Subsequently, we expressed the *hRAD54* and the *hRAD54*^{K189R} constructs in a baculovirus expression system and purified both encoded proteins. Since the Rad54 protein has sequence motifs also found in proteins that bind and hydrolyze ATP, we examined whether purified hRad54 has ATPase activity. We found that efficient ATPase activity was absolutely dependent on the presence of double-stranded (ds) DNA. The hRad54^{K189R} protein could not hydrolyze ATP. Analysis of the rate of ATP hydrolysis by hRad54 showed that hRad54 exhibits a turnover rate within the range of DNA helicases involved in recombination and repair. Therefore, five distinct DNA substrates were tested in helicase assays, since DNA helicases differ in their substrate specificity. Under the conditions tested, we have not detected any helicase activity of hRad54.

In **Chapter 7**, the cellular behavior of the mRad54 protein and the interaction between hRad54 and dsDNA are described. During homologous recombination, Rad51 mediates joint molecule formation between a single-stranded (ss) DNA region and homologous duplex DNA. Rad51 initiates pairing by forming a nucleoprotein filament on the DNA. Recently, it has been shown that ScRad54 stimulates the rate of this pairing. However, the role or mechanism of ScRad54 in stimulating this process is unclear. The interaction of hRad54 with dsDNA was examined using a topological assay. In the presence of ATP, hRad54 introduces negative supercoils in plasmid DNA, most likely due to unwinding of the DNA double helix. To study the cellular behavior of mRad54, we generated mouse ES cells in which one of the *mRAD54* alleles was fused to a nucleotide sequence at the carboxy-terminus encoding for a HA tag. Placing the HA-tagged *mRAD54* under its

endogenous promoter ensured properly regulated expression of the protein. Specific detection of the mRad54-HA protein on immuno-blots could now be performed using α-HA antibodies. Cells expressing only the HA-tagged mRad54 displayed the same IR resistance as cells expressing wild-type mRad54, implying that the HA tag did not interfere with the biological function of the Rad54 protein. IR treatment of the ES cells did not result in a dose- or time- dependent difference in the mRad54 levels as determined by immuno-blot analysis. However, although IR did not change the steady-state level of mRad54, a dramatic redistribution of the protein in the cells was seen by immunofluorescence. While we could not detect mRad54 with immunofluorescence before IR, the protein concentrates in bright nuclear foci after treatment with IR. Interestingly, upon irradiation mRad54 foci colocalize with mRad51 foci. Foci induction of mRad54 in ES cells was also seen after treatment of the cells with mitomycin C and methyl methanesulfonate (MMS), but not UV-light, which correlates to the sensitivities of *mRAD54*$^{-/-}$ ES cells to mitomycin C, MMS, and to their resistance to UV-light. Immunoprecipitation experiments indicate that the two proteins form a stable complex, but only upon irradiation. In addition, we uncovered a role for mRad54 in mRad51 foci formation, since mRad51 does not form IR-induced nuclear foci in *mRAD54*$^{-/-}$ ES cells.

SAMENVATTING VOOR NIET-INGEWIJDEN

DNA kan worden beschouwd als een enorm databestand dat de complete instructie bevat voor het maken van alle eiwitten die een cel ooit nodig heeft. DNA bestaat uit twee lange, gepaarde strengen die samen de zogenaamde "dubbele helix" vormen. Elke streng bestaat weer uit miljoenen bouwstenen, waarvan de basen een belangrijk onderdeel zijn. Alhoewel er maar vier verschillende basen (adenine, thymidine, cytosine en guanine) in het DNA voorkomen, bepaalt juist de volgorde van de basen de specifieke informatie die het DNA bevat. Dit is te vergelijken met de letters van het alfabet die gecombineerd kunnen worden tot woorden en hele zinnen. DNA bevindt zich in de kern van iedere cel. Behalve de rode bloedcellen, bevat iedere cel in een menselijk lichaam dezelfde informatie, opgeslagen als DNA. Iedere cel heeft 46 dubbelstrengs DNA moleculen, die afzonderlijk de chromosomen worden genoemd. Er zijn twee bijna identieke kopieën van elk chromosoom in de cel. Het DNA bestaat uit vele afzonderlijke segmenten, die genen worden genoemd (tussen de 50.000 en 100.000). Ieder gen bevat een informatie set, die meestal codeert voor één bepaald eiwit. DNA kan vele veranderingen (mutaties) ondergaan, waardoor de codering voor een bepaald eiwit verandert. Het gevolg kan zijn dat het betreffende eiwit niet goed of helemaal niet meer functioneert. Afhankelijk van de specifieke functie van het eiwit heeft dit meer of minder ernstige gevolgen.

DNA dubbelstrengs breuken (DSBs), die kunnen ontstaan als gevolg van bijvoorbeeld röntgenstraling, vormen één van de grootste bedreigingen voor de integriteit van DNA in cellen. Het herstel van deze DSBs is essentieel om te voorkomen dat het chromosomaal DNA fragmenteert of andere veranderingen ondergaat waardoor cellen zich uiteindelijk tot kankercellen kunnen gaan ontwikkelen. Om te begrijpen wat het biologische gevolg is van blootstelling aan röntgenstraling moeten we dus inzicht krijgen in de processen die voor het herstel van deze DSBs zorgen. Bovendien vraagt het veelvuldige gebruik van röntgenstraling in anti-kanker therapieën om meer inzicht in DSB herstel. Veel genen, die betrokken zijn bij DSB herstel zijn de afgelopen jaren geïdentificeerd. De analyse van de functie van de eiwitten die door deze genen worden gecodeerd is daarentegen nog lang niet volledig.

In **hoofdstuk 1** wordt de huidige kennis omtrent de rol en het moleculaire mechanisme van de verschillende DSB herstel systemen bediscussieerd. Het bestaan van verschillende systemen voor het herstel van DSBs is waarschijnlijk een weerspiegeling van het extreme gevaar van DSBs voor de cel. De twee belangrijkste DSB herstel systemen zijn "homologe recombinatie" en "DNA eind-verbinding". Met behulp van homologe recombinatie kunnen DSBs foutloos worden gerepareerd doordat een tweede onbeschadigde kopie van een overeenkomstige DNA sequentie wordt gebruikt als een soort matrijs voor het herstel van de DSB. Herstel van DSBs door middel van DNA eind-verbinding

vereist geen matrijs en daardoor is DSB herstel via DNA eind-verbinding niet altijd zonder fouten. Het belangrijkste verschil tussen homologe recombinatie en DNA eind-verbinding is dan ook de nauwkeurigheid van het DSB herstel. Analyse van zoogdier-cellen die gevoelig zijn voor röntgenstraling is van cruciaal belang geweest voor de identificatie van DSB herstel genen. De geïsoleerde genen bleken allemaal betrokken bij DSB herstel door middel van DNA eind-verbinding. Recentelijk is echter gebleken dat genen betrokken bij DSB herstel door middel van homologe recombinatie zijn geconserveerd van gist tot zoogdieren. De functie van de gecodeerde eiwitten betrokken bij homologe recombinatie is dan ook waarschijnlijk belangrijk in de mens.

Eén van de belangrijkste genen betrokken bij DSB herstel door middel van homologe recombinatie in de bakkersgist *Saccharomyces cerevisiae* is het *RAD54* gen. **Hoofdstuk 2 tot en met 7** beschrijft de karakterisering van muizen en cellen waarin één gen (*mRAD54* genaamd) betrokken bij DSB herstel is uitgeschakeld. Verder worden de initiële karakterisering en de cellulaire eigenschappen van het muize en humane Rad54 eiwit beschreven.

In **hoofdstuk 2** wordt de identificatie en karakterisering beschreven van het humane en muize *RAD54* gen dat homoloog is aan het gist *RAD54* gen. Door het humane *RAD54* gen tot expressie te brengen in gist *RAD54* mutanten laten we vervolgens zien dat de functie van het Rad54 eiwit is geconserveerd van gist tot zoogdieren.

In **hoofdstuk 3** wordt de isolatie en uitgebreide karakterisering van het *mRAD54* gen beschreven. De organisatie van de *mRAD54* gen werd bepaald om zogenaamde "gen-onderbrekings" constructen te kunnen maken waarmee het *mRAD54* gen heel nauwkeurig uitgeschakeld kan worden in muizen en cellen.

In **hoofdstuk 4** wordt beschreven hoe we muizen en embryonale stam (ES) cellen hebben gemaakt waarin het *RAD54* gen is uitgeschakeld (*mRAD54*$^{-/-}$). Door deze *mRAD54*$^{-/-}$ muizen en *mRAD54*$^{-/-}$ ES cellen zorgvuldig te analyseren proberen we vervolgens de rol van het Rad54 eiwit in deze muizen en cellen te ontrafelen. Zo hebben we de gevoeligheid voor verschillende stoffen die het DNA kunnen beschadigen en de efficiëntie van homologe recombinatie in *mRAD54*$^{-/-}$ ES cellen bepaald. Aangezien gist *RAD54* mutanten gevoelig zijn voor röntgenstraling, bepaalden we ook de röntgen-gevoeligheid van de *mRAD54*$^{-/-}$ ES cellen. *mRAD54*$^{-/-}$ ES cellen blijken ongeveer 2 maal gevoeliger dan normale ES cellen voor röntgenstraling. Vervolgens laten we zien dat *mRAD54* nodig is voor efficiënte homologe recombinatie. Om de biologische effecten van het uitschakelen van *mRAD54* te bepalen op het hele organisme werden *mRAD54*$^{-/-}$ muizen gegenereerd. *mRAD54*$^{-/-}$ muizen bleken gezond en zonder grote afwijkingen.

In **hoofdstuk 5** wordt de uitgebreide karakterisering van *mRAD54*$^{-/-}$ muizen beschreven. Volwassen *mRAD54*$^{-/-}$ muizen bleken niet gevoelig te zijn voor röntgenstraling. In het embryonale stadium (dag 3.5 na bevruchting) bleken *mRAD54*$^{-/-}$ muizen echter wel gevoelig te zijn voor röntgenstraling. Aangezien zowel ES cellen als cellen in dit embryonale stadium nog ongedifferentieerd zijn,

bepaalden we ook de röntgen-gevoeligheid van een gedifferentieerd celtype, zoals *mRAD54*$^{-/-}$ embryonale fibroblast-cellen. *mRAD54*$^{-/-}$ embryonale fibroblast-cellen bleken 1.4 maal gevoeliger voor röntgenstraling en 2 maal gevoeliger voor mitomycine C. Mitomycine C is een anti-tumor antibioticum en de anti-kanker werking ervan berust op de binding van mitomycine C aan het DNA van de kankercellen door vorming van een complex tussen beide DNA strengen, waardoor de kankercellen afsterven. Verder bleek ook dat het proces van homologe recombinatie in *mRAD54*$^{-/-}$ embryonale fibroblasten minder efficiënt was dan in normale fibroblasten. Het belang van het mRad54 eiwit in volwassen muizen werd bepaald door de gevoeligheid van de volwassen *mRAD54*$^{-/-}$ muizen te bepalen voor mitomycine C. *mRAD54*$^{-/-}$ muizen bleken ongeveer 1.5 maal gevoeliger voor mitomycine C dan normale muizen. Bovendien bleek het aantal chromosomale afwijkingen in bloedcellen als gevolg van de mitomycine C behandeling groter te zijn in *mRAD54*$^{-/-}$ muizen. De conclusie is dat Rad54-afhankelijke homologe recombinatie bijdraagt tot de bescherming tegen röntgenstraling en mitomycine C schade vroeg in de ontwikkeling en alleen tegen mitomycine C schade in de volwassen muis. Aangezien DNA eind-verbinding specifiek bescherming biedt tegen röntgenstraling en niet tegen mitomycine C schade betekent dit waarschijnlijk dat homologe recombinatie en DNA eind-verbinding een overlappende rol hebben in bescherming tegen röntgenstraling. Bescherming tegen mitomycine C schade is echter specifiek afhankelijk van homologe recombinatie.

In **hoofdstuk 6** wordt een start gemaakt met de karakterisering van het hRad54 eiwit. Nadat het Rad54 eiwit gezuiverd was konden we beginnen met het bepalen van de biochemische eigenschappen, zoals beschreven in hoofdstuk 7.

In **hoofdstuk 7** wordt beschreven wat er in de cellen met het Rad54 eiwit gebeurt na bestraling en welke invloed het Rad54 eiwit heeft op dubbelstrengs DNA. Tijdens het proces van homologe recombinatie wordt er onder invloed van een ander belangrijk eiwit betrokken bij homologe recombinatie, Rad51, een complex gevormd tussen het enkelstrengs beschadigde DNA en een tweede kopie van onbeschadigd dubbelstrengs DNA. Dit wordt geïnitieerd door Rad51, dat een filament vormt op het enkelstrengs DNA. Onlangs is aangetoond dat Rad54 dit proces stimuleert, de manier waarop is echter onduidelijk. Met behulp van het gezuiverde Rad54 eiwit laten we zien dat het Rad54 eiwit de DNA dubbele helix kan ontwinden. Vervolgens laten we zien dat het Rad54 eiwit, dat in onbestraalde cellen niet te zien is, na röntgenstraling een herverdeling ondergaat en samen met het Rad51 eiwit zichtbaar wordt als stippen in de kern. Uit biochemische experimenten bleek vervolgens dat het Rad51 eiwit en het Rad54 eiwit een stabiel complex vormen na behandeling van de cellen met röntgenstraling. Bovendien bleek de vorming van Rad51 stippen afhankelijk te zijn van Rad54, aangezien Rad51 geen stippen vormt in bestraalde *mRAD54*$^{-/-}$ ES cellen. De conclusie is dat het Rad54 de dubbele helix ontwindt en daardoor waarschijnlijk bijdraagt tot het stimuleren van de uitwisseling van het beschadigde enkelstrengs DNA met het onbeschadigde dubbelstrengs DNA door het Rad51 eiwit.

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CURRICULUM VITAE

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1982-1988	Gymnasium-β aan de Stedelijke Scholengemeenschap Maastricht
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1993-1994	Wetenschappelijk onderzoeksmedewerker in dienst van het Rijksinstituut voor de Volksgezondheid en Milieu-hygiëne, Laboratorium voor Carcinogenese en Mutagenese.
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