

Enhanced Genomic Instability and Defective Postreplication Repair in *RAD18* Knockout Mouse Embryonic Stem Cells

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In lower eukaryotes, Rad18 plays a crucial role in postreplication repair. Previously, we isolated a human homologue of *RAD18* (*hRAD18*) and showed that human cells overexpressing hRad18 protein with a mutation in the RING finger motif are defective in postreplication repair. Here, we report the construction of *RAD18*-knockout mouse embryonic stem cells by gene targeting. These cells had almost the same growth rate as wild-type cells and manifested phenotypes similar to those of human cells expressing mutant Rad18 protein: hypersensitivity to multiple DNA damaging agents and a defect in postreplication repair. Mutation was not induced in the knockout cells with any higher frequencies than in wild-type cells, as shown by ouabain resistance. In the knockout cells, spontaneous sister chromatid exchange (SCE) occurred with twice the frequency observed in normal cells. After mild DNA damage, SCE was threefold higher in the knockout cells, while no increase was observed in normal cells. Stable transformation efficiencies were ~20-fold higher in knockout cells, and gene targeting occurred with ~40-fold-higher frequency than in wild-type cells at the Oct3/4 locus. These results indicate that dysfunction of Rad18 greatly increases both the frequency of homologous as well as illegitimate recombination, and that *RAD18* contributes to maintenance of genomic stability through postreplication repair.

Exposure of cells to UV light, certain chemicals, or other mutagens can inflict lesions in the DNA. However, cells are normally equipped with repair mechanisms whereby the lesion can be removed efficiently. In the event that a lesion is not removed by base or nucleotide excision, or if the DNA replication machinery happens to meet the lesion before repair, the replication machinery stalls at the lesion, causing a gap opposite the site of damage in the newly synthesized strand. Cell death may be imminent unless the gap can be filled. Gap filling is a function of postreplication repair (PRR), a function conserved in diverse species.

In *Escherichia coli*, proteins involved in PRR are encoded by genes belonging to the SOS regulon and are induced in response to the appearance of single-stranded genomic DNA following DNA damage (11). DNA gaps are filled mainly by recombination in which the RecA and RecFOR complex promotes resolution of the stalled replication fork by allowing damage bypass via template-switching (8, 9; T. Kogoma, Letter, Proc. Natl. Acad. Sci. USA **94**:3483-3484, 1997). However, when DNA damage is severe, another mode of PRR, named translesion synthesis is carried out by an error-prone type DNA polymerase, such as UmuD'₂C (10, 42).

In the yeast *Saccharomyces cerevisiae*, genes belonging to the *RAD6* epistasis group are responsible for the PRR pathway (8, 11, 33). *RAD6*, *RAD18*, *RAD30*, *RAD5*, *REV3*, and *REV7* are major members of this group. DNA gaps caused by replication

stalling are filled by translesion synthesis either in an error-free or an error-prone mode depending on the context of the DNA damage (19). In UV-induced PRR, error-free and error-prone types of translesion synthesis are carried out by DNA polymerase η (Rad30) and DNA polymerase ζ (a heterodimer of Rev3 and Rev7), respectively (19, 27, 29). *RAD6* and *RAD18* play a critical role in controlling PRR, and one of the phenotypes of *rad6* and *rad18* mutants is a high susceptibility to lethal effects by various DNA damaging agents (11, 23, 32). Rad18 protein binds to single-stranded DNA and forms a tight complex with Rad6 protein, a ubiquitin-conjugating enzyme (E2) (6, 7). Thus, it is proposed that Rad18 protein recruits Rad6 protein at replication-stalling sites by binding to single-stranded regions and the Rad6 protein in turn ubiquitinates certain target proteins to be degraded in the PRR process (6, 7). A homologue of *RAD18* has been identified from the filamentous fungus, *Neurospora crassa*. This gene, *uvs-2*, encodes a protein which shares partial homology to Rad18 and interacts with the Rad6 homologue, MUS8 (40).

DNA gaps are also filled by recombination, but the pathway is normally down-regulated by SRS2 (8, 12, 37), which encodes a DNA helicase with 3' to 5' polarity (35). Hypersensitivity to DNA damaging agents in *rad18* and *rad6* mutants is suppressed by the *srs2* mutation (1, 37). Suppression by *srs2* requires *RAD51*, *-52*, *-54*, *-55*, or *-57* genes which function in homologous recombination (36, 37). Thus, it is hypothesized that Srs2 channels lesions into the Rad6-dependent PRR pathway, preventing unfavorable recombinational repair (8, 37).

In mammals, two homologs of the *RAD6* genes, *HR6A* and *HR6B* and a single *RAD18* gene are present (17, 39, 41). The

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HR6B-knockout mice manifest male sterility but otherwise are normal (5, 34). Recently, it was found that human polymerase η is a homologue of yeast Rad30, and that polymerase η is mutated in patients having the variant form of the hereditary photosensitive and cancer-prone disease xeroderma pigmentosum (XP-V) (16, 26). XP-V cells exhibit a feature typical of defective PRR in that the size of newly replicated DNA is shorter than that in normal cells after UV-irradiation (24). These facts indicate the importance of error-free translesion synthesis for maintaining genomic integrity. We cloned the human *RAD18* gene (*hRAD18*) and showed that the hRad18 protein interacts with both the hHR6A and hHR6B proteins (39). Furthermore, human cells expressing Rad18 with a mutation in the RING finger motif are defective in PRR and become sensitive to multiple DNA damaging agents (39). However, in mammalian PRR, both the role of Rad18 and recombination-mediated bypass of DNA damage remain to be determined.

In this study, in order to understand the molecular mechanism of PRR in mammals, we prepared mouse *RAD18*-knockout embryonic stem (ES) cells by gene targeting. These cells were sensitive to multiple DNA damaging agents and were defective in PRR. *RAD18* deficiency enhanced sister chromatid exchange (SCE), stable transformation, and gene targeting, indicating that *RAD18* is required for preclusion of illegitimate recombination, and is thus a key component of systems ensuring genomic stability.

MATERIALS AND METHODS

Cell culture and plasmids. Wild-type ES cells (E14), and *RAD18*^{-/-} ES cells were cultured in Glasgow modification of Eagle medium supplemented with 10% fetal calf serum. Leukemia inhibitory factor was added to the medium to prohibit differentiation of the ES cells. A plasmid containing mouse *RAD18* cDNA (41) was obtained from the IMAGE Consortium (clone ID 535214). The *RAD18* targeting construct was prepared using *RAD18* genomic DNA and the pU-16 plasmid (3). pCAGGS-CRE was used for transient expression of the CRE recombinase in hetero-knockout ES cells (2). Stable transformation frequencies were determined by counting drug-resistant colonies using plasmids, pPGKpuro (3) and pGTIREShphA (30). Targeting frequency was evaluated with the Oct3/4 targeting plasmid Oct3/4IRESshp (30).

Generation of *RAD18*-deficient ES clones. Mouse *RAD18* genomic DNA was isolated from a mouse genomic library, 129/SvJ (Stratagene), by using mouse *RAD18* cDNA as a probe. *RAD18* targeting was performed by a two-step integration of a LacZ-neomycin (β geo) cassette to the *RAD18* genome. In the first step, one allele of the *RAD18* gene was disrupted by integration of an internal ribosome entry site (IRES) β geo-polyA cassette at the *AvrII* restriction enzyme site in the exon of the *RAD18* genomic DNA (Fig. 1A). Then the pCAGGS-CRE plasmid was used to remove the IRES β geo portion by transient expression of the CRE recombinase in the cells, leaving a polyA portion of the vector in the targeted exon. In the second step, the other allele was disrupted using the same targeting construct used in the first step.

Immunocytochemistry. Glutathione *S*-transferase (GST)-mRad18 (Met-373 to Gln-509) fusion protein was purified from *E. coli* extract through a glutathione-agarose column, and then PreScission protease (Amersham) was used to remove the GST portion. Polyclonal antiserum was raised by immunizing rabbits with the cleaved fragments spanning the C terminus of mRad18 (Met-373 to Gln-509). The antiserum was used at a dilution rate of 300. Cells growing on coverslips were prefixed with 3.7% formaldehyde for 2 min at room temperature, washed with phosphate-buffered saline, and then fixed with 80% methanol for 10 min at -10°C. The cells were stained with the diluted antiserum for 30 min at room temperature, washed vigorously, and then stained with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (Cappel) for another 30 min at room temperature.

Assessment of newly synthesized DNA. PRR was evaluated by the method described elsewhere (24) with minor modifications. Briefly, actively growing cells (10^5 cells per dish) were irradiated with UV at 4 J/m², incubated for 30 min, and

then pulse labeled with 0.93 MBq/ml [methyl-³H]thymidine for 15 min. The cells were lysed with a solution containing 0.2 M NaOH and 20 mM EDTA, and the lysates were irradiated with X rays at 20 Gy and centrifuged by alkaline sucrose density as described previously (39). Control cells were treated in the same manner but without UV irradiation.

Immunoprecipitation. Immunoprecipitation was done by the method described elsewhere (39). Briefly, COS-7 cells were transfected with 10 μ g of plasmid by electroporation employing an Electro Cell Manipulator (BTX) and cultured for 48 h. Cells were lysed in 1 ml of lysis buffer and disrupted by sonication. The cleared cell lysate was prepared by centrifugation and mixed with 2 μ g of an anti-T7 monoclonal antibody (Novagen), or an anti-GST polyclonal rabbit antibody (Santa Cruz) for 1 h at 4°C. After addition of 50 μ l of a slurry of protein-G Sepharose beads (Pharmacia), the lysate was mixed for an additional hour at 4°C. Fifty percent of the bound fraction was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 or 12.5% polyacrylamide). Separated proteins were identified with the enhanced chemiluminescence (ECL) System (Amersham) using either anti-mRad18 antibody or anti-MUS8 rabbit serum. An anti-*N. crassa* MUS8 antiserum, which cross-reacts well with mouse HR6A and HR6B proteins, was raised in rabbits by immunizing with GST-MUS8 fusion protein.

Measurement of mutation frequency. Mutation frequency was determined by counting ouabain-resistant colonies after treatment with or without DNA damaging agents. In brief, cells (2×10^6) were cultured for 20 h and exposed to different DNA damaging agents (0.7 to 1.5 J of UV irradiation/m² or 50 to 100 μ g of methyl methanesulfonate (MMS)/ml for 1 h). The cells were incubated for 2 days. They were harvested, inoculated to new 100-mm-diameter dishes (2×10^6 cells/dish; 10 to 14 dishes). Ouabain was added to the medium at a final concentration of 2 mM and incubated for 2 weeks with medium change every 3 days. The surviving colonies were fixed with 3.7% formaldehyde and stained with 3% Giemsa solution.

SCE analysis. SCE analysis was done by the method described elsewhere (31). In brief, cells (10^6) were cultured in the dark for approximately two cycles in medium containing 15 μ M bromodeoxyuridine (BrdU). Colcemid was included at a concentration of 0.05 μ g/ml for the final 2 h. Cells were harvested and treated with 75 mM KCl for 20 min at room temperature and then fixed with methanol-acetic acid (3:1) for 30 min. The cells were washed once with the fixative and then suspended in a small volume of the fixative. The cell suspension was dropped onto ice-cold wet glass slides and air dried. The cells on the slides were incubated with Hoechst 33258 (5 μ g/ml) in H₂O for 20 min, rinsed with MacIlvaine solution (164 mM Na₂HPO₄, 16 mM citric acid [pH 7.0]) and covered with a coverslip. Cells were exposed to black light ($\lambda = 325$ nm) at a distance of 1 cm for 30 min and washed with water for 5 min. They were stained with 3% Giemsa solution at pH 6.8 for 10 min, and examined with a light microscope. To evaluate the effects of DNA damaging agents on SCE, cells were treated with these agents just before labeling in the medium containing BrdU.

Assessment of stable transformation. ES cells were transfected with pPGK-puro, pGTIREShphA, or Oct3/4IRESshp plasmids by electroporation and then cultured in medium containing puromycin (1 μ g/ml) or hygromycin B (0.2 mg/ml) (Wako Japan) for 2 weeks. The frequency of stable transformation was assessed by the frequencies of antibiotic-resistant colonies.

Evaluation of gene targeting. The targeting efficiency of ES cells was evaluated with the construct Oct3/4IRESshp. Single disruption of Oct3/4 has no effect on viability (30). After electroporation, antibiotic-resistant clones were selected in a medium containing hygromycin B (0.2 mg/ml) for 2 weeks. Surviving colonies were transferred and propagated individually. Genomic DNA from the individual clones was cleaved with *EcoRI* and analyzed for targeting by probing the Southern blot with Oct3/4 DNA.

RESULTS

Establishment of *RAD18*^{-/-} ES cells. *RAD18* knockout ES cells were prepared in two steps by sequentially knocking out both alleles with the same targeting vector as described in Materials and Methods. The target exon encodes part of the Rad18 protein between the RING finger motif and the zinc finger (Fig. 1A). Single and double knockouts of *RAD18* alleles were confirmed by Southern blotting with multiple ES clones (Fig. 1B). As expected, the amounts of *RAD18* mRNA in wild-type, single-knockout, and double-knockout ES cells were roughly 2:1:0, respectively (Fig. 1C). However, the amount of

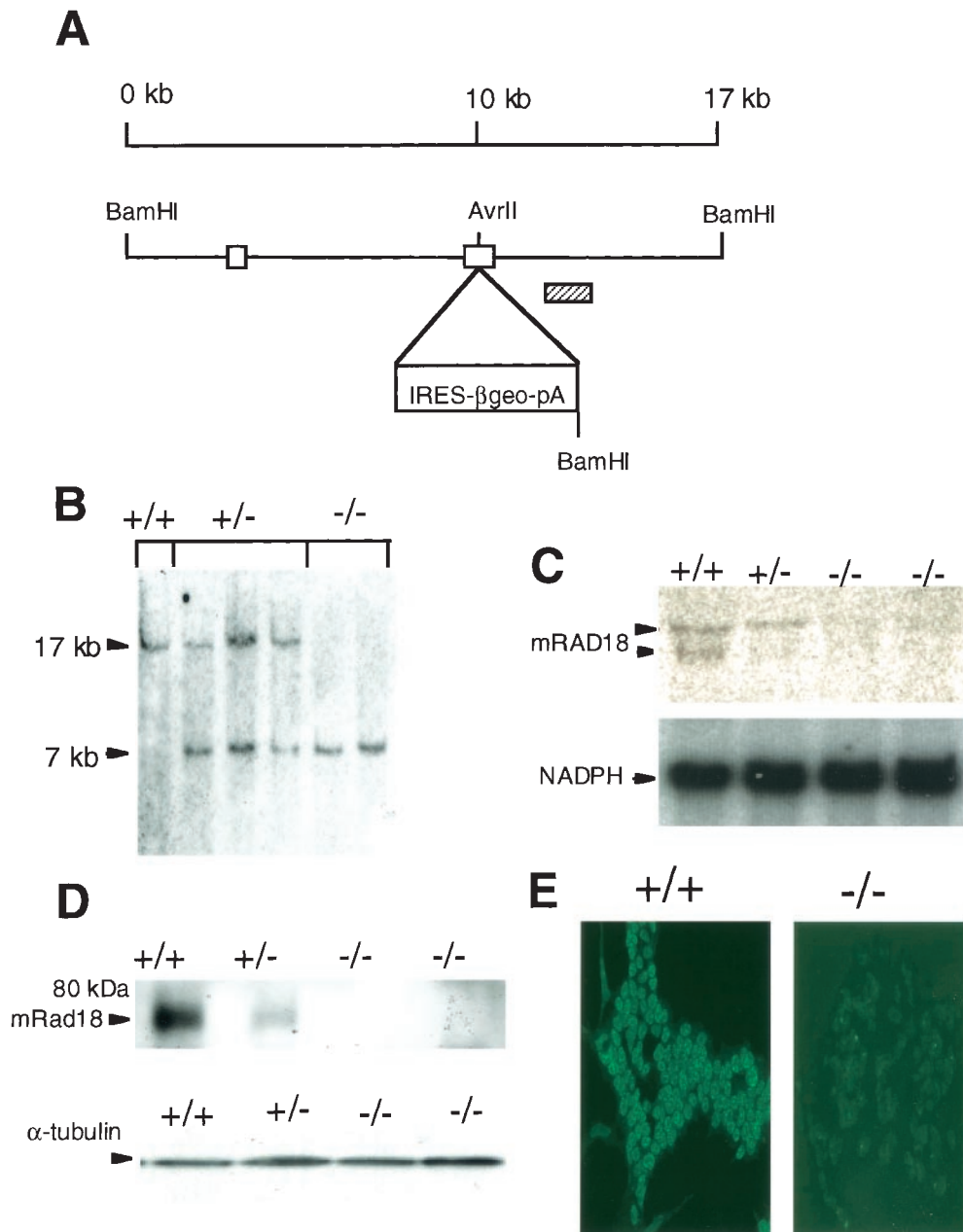


FIG. 1. Generation of mouse *RAD18*^{-/-} clones. (A) Schematic representation of disruption constructs. The IRES- β geo-polyA cassette was inserted at the *AvrII* site of the exon encoding amino acids Thr-90 to Lys-201. The hatched box indicates sequence used to probe Southern blots. (B) Southern blot analysis. *Bam*HI-digested genomic DNA prepared from different ES clones (first lane, wild type; second to fourth lanes, *RAD18*^{+/-}; fifth and sixth lanes, *RAD18*^{-/-}) was hybridized with the probe. (C) Northern blot analysis. Total RNA from different ES clones (first lane, wild type; second lane, *RAD18*^{+/-}; third and fourth lanes, *RAD18*^{-/-}) was hybridized with an *mRAD18* cDNA probe that corresponds to the N terminus of the gene product. Multiple *mRAD18* mRNA bands, indicated by arrowheads, were detected. NADPH mRNA is shown as a control. (D) Western blot analysis. Cell lysates from different ES clones were immunoblotted with a polyclonal rabbit antibody against mRad18. Protein levels of α -tubulin in the lysates are shown as a control. (E) Wild-type (left) and *RAD18*^{-/-} ES cells (right) were stained for endogenous Rad18 protein with an anti mRad18 antibody.

Rad18 protein in *RAD18*^{+/-} single-knockout ES cells was considerably less than a half of that of wild-type ES cells as revealed by Western blotting, and no Rad18 protein was detected in the *RAD18*^{-/-} ES cells (Fig. 1D). As reported elsewhere (39), immunostaining showed that Rad18 was localized in the nuclei of wild-type ES cells whereas no positive signal was observed in the *RAD18*^{-/-} ES cells (Fig. 1E).

To examine the effect of *RAD18* disruption on cell proliferation, we monitored the growth rates of wild-type and *RAD18*^{-/-} ES cells. *RAD18*^{-/-} cells proliferated at almost the same rate as the wild-type cells, having a doubling time of 16 h (Fig. 2).

Defective PRR in *RAD18*^{-/-} ES cells. The sensitivity of *RAD18*^{-/-} ES cells to a variety of DNA damaging agents was

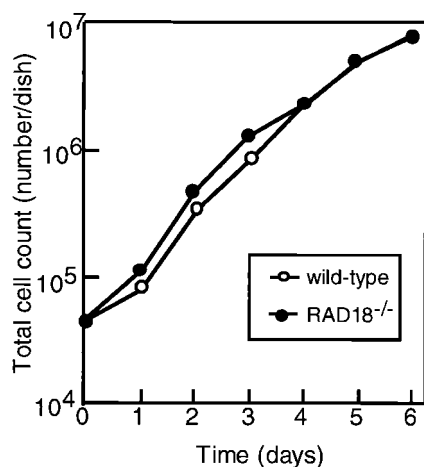


FIG. 2. Growth curves of wild-type cells (○) and *RAD18*^{-/-} cells (●). Cells were counted with a hemacytometer at the indicated times. The data show typical results of three independent experiments.

determined by the colony formation assay. The knockout ES cells showed moderate sensitivities to UV, MMS, mitomycin C (MMC), and cisplatin compared to wild-type ES cells (Fig. 3A to D). However, no difference in sensitivity to X rays was observed (Fig. 3E). Increased sensitivity to multiple types of DNA damaging agents is a characteristic of the *rad18* yeast mutants defective in PRR, and is not observed in XP cells defective in nucleotide excision repair. To confirm there are defects in PRR in *RAD18*^{-/-} ES cells, we measured the sizes of replicated DNA after UV-irradiation by alkaline sucrose density gradient centrifugation. There were smaller fragments of DNA in irradiated *RAD18*^{-/-} ES cells in comparison to those in nonirradiated cells, while in normal ES cells the profiles more closely resembled those of nonirradiated cells (Fig. 4).

Association of mouse Rad18 protein with mouse Rad6 protein. To determine whether mouse Rad18 protein (mRad18) interacts with mouse Rad6 protein (mHR6), we performed immunoprecipitation experiments. To make detection easier, GST-tagged mRad18 plasmid and T7-tagged mRad6 plasmid were transfected into COS-7 monkey cells, and tagged proteins were overproduced there. GST-mRad18 was precipitated by T7-tag-specific monoclonal antibody only when T7-*mHR6A* or T7-*mHR6B* was cotransfected with GST-*mRAD18* (Fig. 5A, lanes 3 and 4). Inversely, T7-mHR6A or T7-mHR6B was precipitated by GST-tag-specific polyclonal antibody only when GST-*mRAD18* was cotransfected with T7-*mHR6A* or T7-*mHR6B* (Fig. 5B, lanes 3 and 4). These results indicate that mRad18 and mHR6A/mHR6B can physically associate with each other in transfected mammalian cells.

Mutation frequencies induced by mutagens in *RAD18*^{-/-} ES cells. To evaluate the effects of *RAD18* disruption on the frequency of induced mutation, *RAD18*^{-/-} ES cells were treated with either UV or MMS, and viable colonies growing in medium containing ouabain were scored. Ouabain binds competitively at the K⁺ ion-binding site of the Na⁺-K⁺ pump on the plasma membrane, inhibiting ATP production (21). Under normal culture conditions, there was no significant difference in the mutation frequency of normal and *RAD18*^{-/-} ES cells,

with few colonies per 10⁷ cells (Fig. 6). However, upon a low dose of UV-irradiation (0.7 J/m²), mutation frequencies in wild-type ES cells increased approximately 25-fold above the background level, while in *RAD18*^{-/-} ES cells it increased approximately 13-fold. Mutation frequencies increased in both cell types with an increase in UV dose to 1.5 J/m², but the frequency in wild-type ES cells remained higher than that of *RAD18*^{-/-} ES cells. Similarly, mutation frequencies of both wild-type and *RAD18*^{-/-} ES cells increased following MMS treatment, but the mutation frequency of *RAD18*^{-/-} ES cells did not exceed that of wild-type cells.

Enhanced sister chromatid exchange in *RAD18*^{-/-} ES cells. SCE is an event of homologous recombination between sister chromatids during DNA replication (38). SCE levels are low in normal cells, but significantly higher than in cells derived from

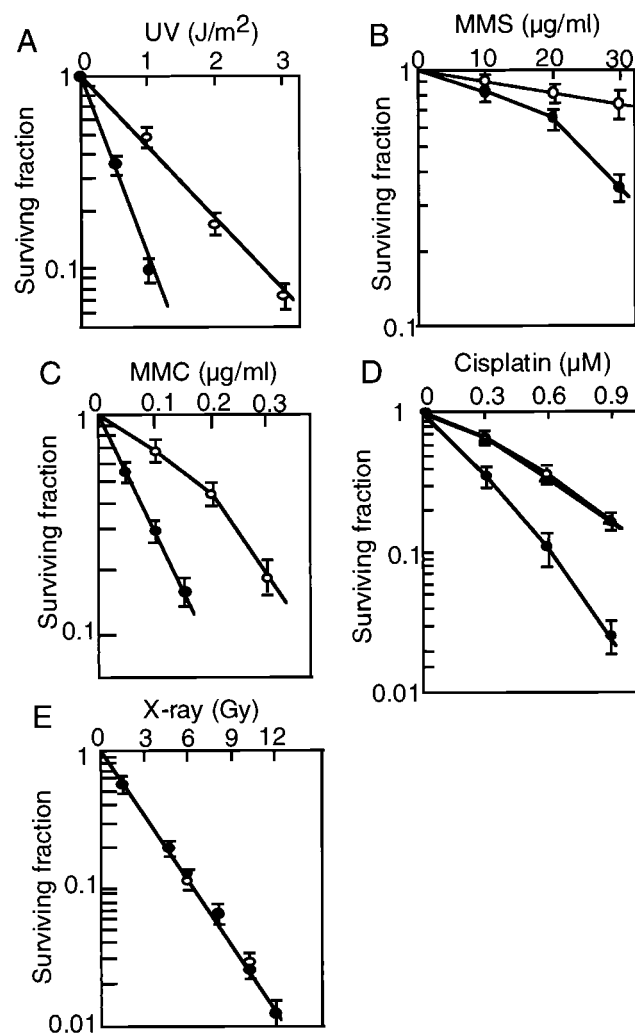


FIG. 3. Sensitivity of *RAD18*^{-/-} ES cells to DNA damaging agents. Cells were irradiated with UV (A), treated with the indicated concentrations of MMS (B) or mitomycin C (MMC) (C) for 1 h, continuously cultured in medium containing cisplatin (D), and irradiated with X-rays (E). Sensitivity was determined by the colony formation assay. Mean values of triplicate dishes are shown with standard deviations (error bars). Symbols: ○, wild-type cells; △, *RAD18*^{+/+} cells; ●, *RAD18*^{-/-} cells.

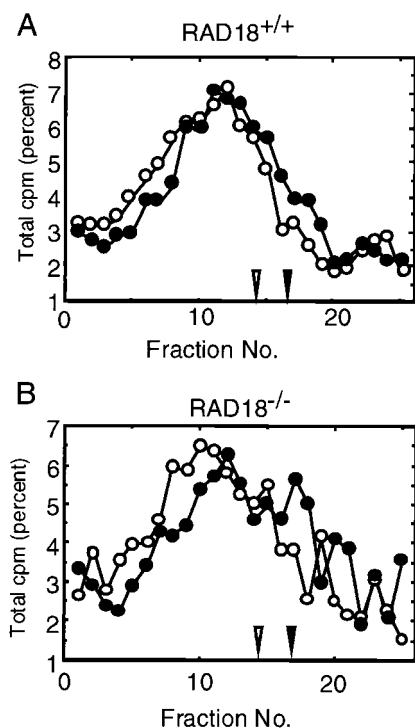


FIG. 4. Impaired PRR of *RAD18*^{-/-} cells. Wild-type ES cells (A) or *RAD18*^{-/-} cells (B) were irradiated with UV (4 J/m²), incubated for 30 min, and then pulse-labeled with [methyl-³H]thymidine for 15 min. Samples were sedimented on 5 to 20% alkaline sucrose gradients from right to left, and the profile of the UV-irradiated cell sample (●) was compared with that of the unirradiated control cell sample (○). The arrowheads indicate the position of size standards: bacteriophage lambda DNA (42 kb) (closed arrowhead) and bacterial artificial chromosome DNA (100 kb) (open arrowhead).

patients with the cancer-prone hereditary disease Bloom's syndrome, which is caused by a defect in *BLM* encoding a DNA helicase belonging to the RecQ family. Hyper-SCE indicates a high incidence of chromosome rearrangements, which reflects genomic instability (11). To investigate whether disruption of *RAD18* affects genomic stability, we measured SCE frequencies with or without genotoxic assaults. Under normal conditions, SCE frequency in *RAD18*^{-/-} ES cells was more than twice that in normal cells (Fig. 7). Following mild treatments with UV, MMC, or MMS, SCE frequencies increased to nearly three times above the control level in *RAD18*^{-/-} ES cells, while such treatments had little effect on wild-type ES cells (Fig. 7B).

Enhanced recombination in *RAD18*^{-/-} ES cells. The frequency of targeted integration and SCE is increased in *BLM*^{-/-} DT40 cells (43). To investigate whether similar effects occur in *RAD18*^{-/-} ES cells, transfection experiments were performed. The frequency of stable transformation was determined by counting colonies grown in selection media with three kinds of plasmids: a plasmid containing a puromycin-resistant gene and two promoterless plasmids containing a hygromycin B-resistant gene. Irrespective of the type of plasmid used, transformation frequencies of *RAD18*^{-/-} ES cells were ~20 times higher than those in wild-type ES cells (Table 1).

To assess targeting efficiency, the promoter-less Oct3/

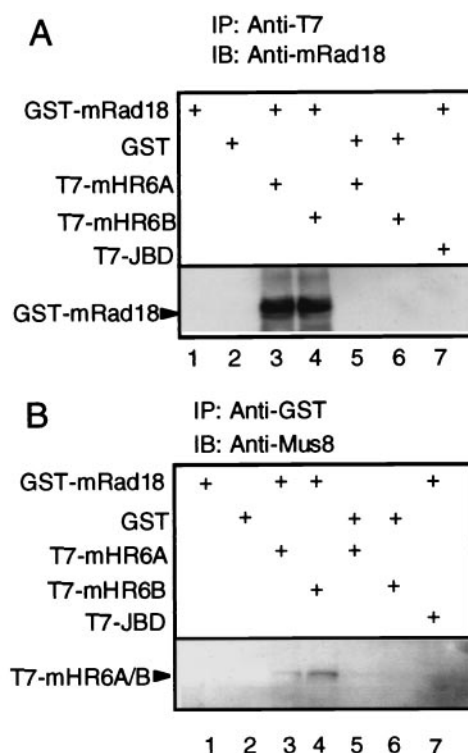


FIG. 5. Interaction of mouse Rad18 protein with mouse Rad6 (mHR6A or mHR6B) protein. Plasmids indicated on the left side were transfected into COS-7 cells in different combinations, and cell lysates were prepared 48 h later. Protein interaction was examined by immunoprecipitation. (A) Proteins immunoprecipitating with an anti-T7 antibody (lanes 1 to 7) were immunoblotted with an anti-*RAD18* antibody. (B) Proteins immunoprecipitating with an anti-GST antibody (lanes 1 to 7) were immunoblotted with an anti-MUS8 (*N. crassa* homologue of *RAD6*) antiserum. JBD, Jun kinase binding domain.

4IRES^{hph} plasmid was transfected into *RAD18*^{-/-} ES cells and into wild-type ES cells. More than 50 hygromycin B-resistant colonies were isolated, and gene targeting at the Oct3/4 locus was determined for individual clones by genomic South-

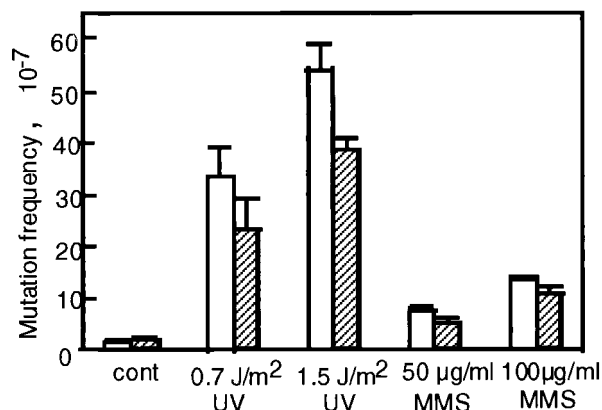


FIG. 6. Damage-induced mutation frequency in *RAD18*^{-/-} ES cells. ES cells were treated with either UV or MMS at the indicated doses. Mutation frequency was determined by the ratio of the numbers of ouabain-resistant colonies to the initial number of viable cells. Mean values of triplicate data are shown with standard deviations (error bars). Open columns, wild-type ES cells; hatched columns, *RAD18*^{-/-} ES cells.

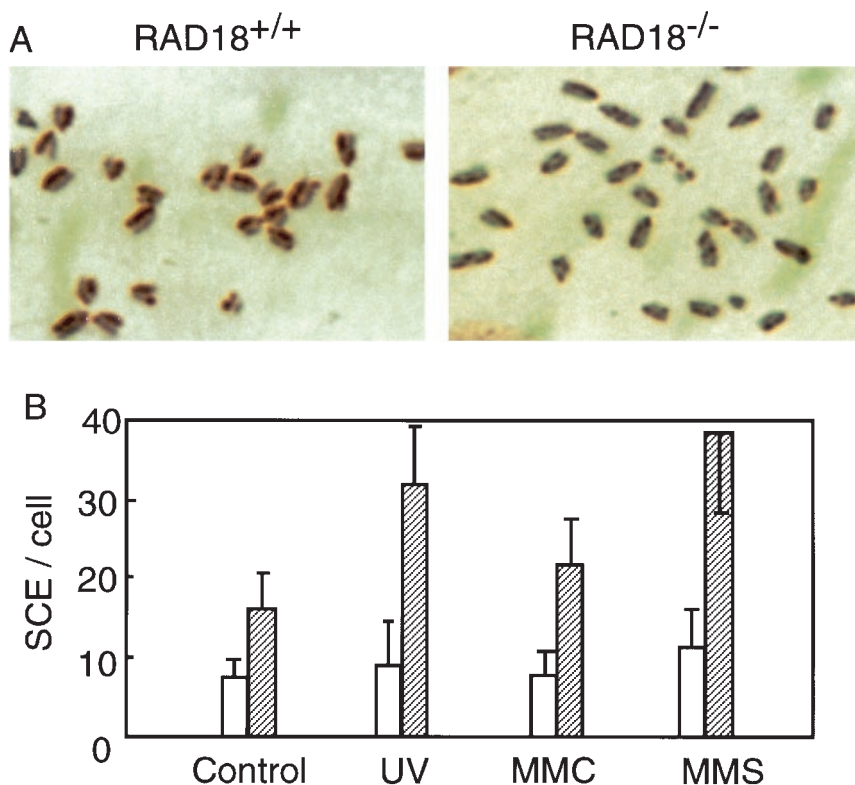


FIG. 7. SCE in *RAD18*^{-/-} ES cells. (A) Differential staining for SCE. Cells were cultured in the presence of BrdU through two cell cycles to enable identification of sister chromatids by differential staining. At left are shown wild-type cells; at right are shown *RAD18*^{-/-} ES cells. (B) Frequencies of SCE in wild-type ES cells and *RAD18*^{-/-} ES cells. Cells were irradiated with UV (0.5 J/m²) or treated with either MMC (0.05 μg/ml) or MMS (25 μg/ml) for 1 h before culture in medium containing BrdU. Control cells remained untreated before labeling with BrdU. The average number of SCE in at least 40 cells is shown with standard deviations. Open columns, wild-type ES cells; hatched columns, *RAD18*^{-/-} ES cells.

ern blotting. Compared to wild-type ES cells, targeting efficiency in *RAD18*^{-/-} ES cells was about twofold higher, with nearly 70% of the drug-resistant clones having targeted integration of plasmid DNA (Table 2). In consequence of the increased rate of both transformation and targeted integration, the overall targeting efficiency in *RAD18*^{-/-} ES cells was about 40 times higher than that of wild-type ES cells. These results indicate that disruption of the *RAD18* gene causes enhancement of both nonhomologous and homologous recombination.

DISCUSSION

In this study, we have established mouse *RAD18*-knockout ES cells by gene targeting and reached the following conclusions. First, Rad18 protein is dispensable for cell viability, since

RAD18^{-/-} ES cells exhibited a similar growth rate to that of wild type cells. Second, *RAD18*^{-/-} ES cells were hypersensitive to a wide variety of DNA damaging agents and defective in PRR. Third, the rate of UV-induced mutagenesis in *RAD18*^{-/-} cells was reduced compared to that in wild type cells. Fourth, frequencies of spontaneous and DNA-damage induced SCE were elevated in knockout cells. Finally, efficiencies of random and targeted integration of exogenous DNA into the genome were also elevated in *RAD18*^{-/-} ES cells. Although our targeting construct is designed to eliminate Rad18 protein with the exception of an N-terminal portion including the RING finger motif, we could neither detect any Rad18 mRNA with a probe corresponding to the N terminus nor any Rad18 protein by either Western blotting or immunostaining. Probably, the truncated form of mRNA is unstable

TABLE 1. Frequency of stable transformation^a

Plasmid	Genotype of host cells		RAD18 ^{-/-} /wild type ratio
	Wild type	RAD18 ^{-/-}	
pPGKpuro	20	400	20
pGTIREShphA	30	590	20
Oct3/4IRESph	30	630	21

^a Wild-type and *RAD18*^{-/-} ES cells (10⁶) were transfected with the individual plasmids, transferred to plates of medium containing appropriate antibiotic (puromycin or hygromycin B), and incubated for 2 weeks prior to the counting of surviving colonies.

TABLE 2. Frequency of targeted integration^a

Expt no.	No. of colonies with targeted integration/total no. tested	
	Wild type	RAD18 ^{-/-}
1	11/27	23/31
2	11/32	15/24
Total (%)	22/59 (37)	38/55 (69)

^a The Oct3/4 allele-targeting construct was transfected into cells. After selection, targeted integration events were determined by Southern blot analysis.

and degraded rapidly. Furthermore, because nuclear import of Rad18 protein requires a nuclear localization signal located in the missing C-terminal portion (unpublished data), we conclude that the knockout ES cells do not contain any functional Rad18 protein, even if a trace amount of the truncated form is synthesized.

The conclusion that our *RAD18*^{-/-} ES cells are defective in PRR is supported by the following lines of evidence, which are characteristics of *rad18* mutants in lower eukaryotes. (i) Mouse Rad18 protein associated with the mouse Rad6 protein in vivo. This is a prerequisite for Rad18 protein to function in the postreplication pathway. (ii) *RAD18*^{-/-} ES cells were sensitive to DNA lesions induced by various DNA damaging agents such as UV, MMS and MMC, and the lesions are repaired by different repair systems. (iii) The size range of newly synthesized DNA in UV-irradiated *RAD18*^{-/-} ES cells was shorter than that of wild type cells, as determined by alkaline sucrose density gradient centrifugation.

These phenotypes of *RAD18*^{-/-} ES cells are similar to those of human cells expressing Rad18 protein mutated in the RING finger motif or antisense *RAD18* mRNA (39). However, sensitivity of the present cells to various DNA damaging agents was mild compared to the *rad18* mutants of lower eukaryotes such as *S. cerevisiae* or *N. crassa*. Previously, we assumed that the mild sensitivity of human cells expressing mutant Rad18 or antisense mRNA was due to incomplete inhibition of Rad18 (39). However, since in the present study, *RAD18*^{-/-} ES cells also showed sensitivity to DNA damage at levels comparable to those of human cells, we conclude that defects in the function of Rad18 in higher eukaryotes result in a mild phenotype compared to lower eukaryotes. Given that the PRR system operates solely on DNA replication, it is plausible that the more severe phenotype of *RAD18* mutation in lower eukaryotes might reflect a higher chance for the DNA replication machinery to meet DNA lesions owing to their more rapid proliferation than higher eukaryotes. Alternatively, some back-up systems such as the Rad5-dependent PRR system or recombination systems might ameliorate the Rad18 defect in higher eukaryotes. The phenotype of cells with multiple defects in these repair systems should help resolve this issue. In lower eukaryotes, *rad18* mutants show moderate sensitivity to ionizing radiation. However, we observed no differences between *RAD18*^{-/-} and wild-type ES cells in their sensitivity to X ray. Currently, we do not know whether this is a general phenotype in higher eukaryotes with defective Rad18, or whether this is a specific phenotype of ES cells. Further studies, for example with other cell types derived from *RAD18* knockout mice, will be required to determine whether the absence of enhanced sensitivity to ionizing radiation in *RAD18*^{-/-} mutants is a peculiarity of ES cells.

In the *RAD18*^{-/-} ES cells, frequency of UV- or MMS-induced mutagenesis was lower than that of wild-type ES cells (Fig. 6). In *S. cerevisiae*, the dramatic increase in mutation in UV-treated cells is also suppressed in *rad18* mutants (4), suggesting that at least a part of DNA damage-induced mutagenesis is *RAD18*-dependent. In *S. cerevisiae*, *RAD18* is required for both error-free and error-prone modes of PRR, as shown by its epistatic relationship with other members of the group, including *RAD30*, *REV3*, and *REV7* (11, 15, 19, 22). While *RAD30*, which encodes DNA polymerase η , functions in an

error-free manner in the repair of UV-induced lesions (27), DNA polymerase ζ , which consists of Rev3 and Rev7, functions in an error-prone manner (29, 32). Probably in ES cells Rad18 is required for the error-prone translesion synthesis mediated by mouse Rev3 and/or Rev7 in response to DNA damage. In contrast to the *rad18* mutants of *S. cerevisiae*, induced mutation frequency in *RAD18*^{-/-} ES cells was higher than the spontaneous level, suggesting the existence of another error-prone translesion synthesis pathway that is independent of Rad18.

In *S. cerevisiae*, *rad18* mutants show elevated levels of spontaneous recombination, ectopic gene conversion, and recombination between direct repeats (25, 36), all of which are dependent on *RAD51*, *RAD52*, and *RAD57* gene products (25). Since Rad18 is involved in repairing single-stranded gaps (13) and maintaining the integrity of single-stranded DNA (ssDNA) and linear DNA created by genotoxic assaults (28), aberrant DNA structures such as single-stranded DNA, double-strand breaks, and gaps may accumulate in *rad18* mutants. These structures are known to be preferential substrates for homologous recombination (9, 14, 18, 20). Enhancement of SCE and gene targeting observed in *RAD18*^{-/-} ES cells (Fig. 7; Table 2) might be caused by accumulation of the aberrant DNA structures either during normal growth or following genotoxic assaults. In addition, frequency of stable transformation was elevated in *RAD18*^{-/-} ES cells (Table 1), suggesting enhancement of nonhomologous recombination. Efficient stable transformation in *RAD18*^{-/-} ES cells might also be triggered by accumulation of the aberrant DNA structures. Whatever the molecular mechanism of recombination may be in mammals, *RAD18*^{-/-} ES cells provide a useful tool for analysis of the Rad6/Rad18 pathway and for establishing stable transformants.

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