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A Role for *RAD51* in the Generation of Immunoglobulin Gene Diversity in Rabbits

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Abstract

Ig VDJ genes in rabbit somatically diversify by both hyperpointmutation and gene conversion. To elucidate the mechanism of gene conversion of IgH genes, we cloned a rabbit homologue of *RAD51*, a gene involved in gene conversion in *Saccharomyces cerevisiae* (yeast), and tested whether it could complement a yeast *rad51* mutant deficient in recombination repair. We found that rabbit *RAD51* partially complemented the defect in switching mating types by gene conversion as well as in DNA double-strand break repair after γ-irradiation. Further, by Western blot analysis, we found that levels of Rad51 were higher in appendix-derived B lymphocytes of 6-wk-old rabbits, a time at which IgH genes diversify by somatic gene conversion. We suggest that Rad51 is involved in somatic gene conversion of rabbit Ig genes.

Antibody diversity is generated by somatic rearrangement of V, D, and J gene segments during maturation of B lymphocytes. These rearrangements result from site-specific DNA recombination that is mediated by several *trans*-acting factors, including RAG1 (1), RAG2 (2), Ku 70/86 (3–5), XRCC4 (6) and DNA-PK_{CS} (7, 8). In response to antigenic stimulation, the rearranged V(D)J genes in mouse and humans undergo further somatic diversification by hyperpointmutation (9–18). In other species, including chickens and rabbits, further somatic diversification occurs by gene conversion as well as by hyperpointmutation (19–24). Although several *cis*-acting factors are known to regulate hyperpointmutation (25–31), little is known about *trans*-acting factors for either somatic gene conversion or hyperpointmutation of Ig genes.

Gene conversion is the nonreciprocal exchange of genetic information that results in an unequal recovery of genetic alleles (32, 33). In *Saccharomyces cerevisiae* (yeast), gene conversion events may result from the recombinational repair of DNA double-strand breaks that is mediated by the *RAD52* epistasis group (34). It is believed that homologous recombination pathways that generate gene conversion events are conserved as a result of the discovery of recombinases that have significant amino acid similarities. Accumulating biochemical and molecular evidence that *S. cerevisiae RAD51* is a structural and functional

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eukaryotic homologue of bacterial *RecA* (35–40), a gene long known to be critical for recombination (41), has stimulated intensive efforts to identify mammalian equivalents of *RAD51*. Eukaryotic homologues to *RecA/RAD51* are now known in several higher species, including chicken (42), mouse (43), and human (44). Biochemical activities of the purified human Rad51, including formation of nucleoprotein filaments with both single-stranded and double-stranded DNA, DNA-dependent ATPase activity (45, 46), pairing homologous DNA molecules (47), and catalyzing strand exchange with a polarity similar to yeast Rad51 (39, 48), suggest that Rad51 may play a role in homologous recombination. However, it is difficult to demonstrate that *RAD51* is involved in recombination in vivo because disruption of the *RAD51* gene leads to lethality of both cell lines and embryos (49, 50).

DNA sequence analysis of diversified rabbit VDJ gene rearrangements and nonrearranged V_H gene segments has shown that gene conversion events occur with the VDJ gene as a recipient and nonrearranged V_H gene segments as donors (22). This process occurs in gut-associated lymphoid tissue, beginning at ~4 wk of age (51–53). We have begun to search for *trans*-acting factors involved in somatic gene conversion of rabbit Ig genes, and, as part of this effort, we have cloned a rabbit *RAD51* homologue. Because of the difficulty in determining, in vivo, the function of *RAD51* in vertebrates, we tested whether rabbit *RAD51* could function in homologous recombination using *rad51* mutant yeast, which are defective in homologous recombination and therefore cannot repair DNA damage caused by ionizing radiation and cannot switch mating types by gene conversion (54–56). Further, to investigate the role of *RAD51* in the generation of Ig gene diversity, we determined whether its expression correlated with the timing and location of somatic gene conversion of IgH genes.

Materials and Methods

Cloning and nucleotide sequence analysis of rabbit RAD51

Thymic cDNA was synthesized and amplified by PCR using as a 5′ primer (5′-ATGGCAATGCAGATGCAGCT-3′) and as a 3′ primer (5′-TCAGTCTTTGGCATCTCCCA-3′), sequences that are homologous to the 5′ and 3′ ends of human *RAD51*, respectively (44). The PCR product was cloned into M13 mp18/mp19 and the nucleotide sequence was determined (57).

Genomic and Northern blot analysis

Genomic DNA was prepared from rabbit liver as described (58), and Southern blot analysis of restricted DNA (10 μg) was performed using the rabbit *RAD51* PCR product as a probe (59). RNA was isolated by density gradient centrifugation in CsCl, and poly(A)$^+$ RNA was purified using a Quick Prepmicro mRNA purification kit (Pharmacia, Piscataway, NJ). For Northern blot analysis, ~3 μg of poly(A)$^+$ RNA was separated by electrophoresis in 1.5% agarose and 1 M formaldehyde gels, and the gels were blotted onto nylon membrane.

Genomic library

A partial *Mbo*I genomic DNA library in EMBL4 (60), prepared from DNA of a V_Ha2 allotype rabbit, was screened using the rabbit *RAD51* cDNA as probe. *RAD51*$^+$ phage

were isolated and characterized by restriction mapping and Southern blot analysis. Individual exons were cloned into M13/mp18 and the nucleotide sequences were determined. A size-selected library of 8 to 10-kb *Eco*RI fragments isolated from liver genomic DNA was constructed in pGEM3 (60) and probed with an exon 1 and exon 2 PCR product by using 5′-ATGGCAATGCAGATGCAGCT-3′ as a 5′ primer and 5′-CAGAATTTTATCAGCTTTTG-3′ as a 3′ primer.

Anti-Rad51 serum

Anti-Rad51 serum was prepared by immunizing rabbits with histidinetagged human Rad51 purified from *Escherichia coli* by ion-exchange chromatography (Qiagen, Santa Clarita, CA). The plasmid encoding the histidine-HsRad51 fusion protein was kindly provided by Charles Radding (Yale University). Serum from an immunized rabbit reacted with a 38-kDa protein (the expected size of Rad51) present in lysates from rabbit, mouse, and chicken cell lines as determined by Western blot analysis, whereas preimmunization serum from the same rabbit did not recognize this protein.

Western blot analysis

IgM^+ and IgL^+ cells were isolated from the appendix by flow cytometry after reaction with biotinylated goat anti-rabbit L-chain followed by FITC-conjugated avidin or with mouse monoclonal anti-rabbit μ-chain followed by FITC-conjugated goat anti-mouse Ig. By reanalysis, the sorted cells were shown to be >95% Ig^+ ($FACStar^{Plus}$, Becton Dickinson, San Jose, CA). Lysates from 1×10^6 purified IgM^+ or IgL^+ cells were electrophoresed on an SDS/10% polyacrylamide gel and blotted to a 0.1 μm nitro-cellulose membrane. Rabbit Rad51 was detected by using rabbit anti-human Rad51 polyclonal antiserum as primary reagent, biotinylated goat anti-rabbit L-chain as secondary reagent, followed by avidin-HRP and enhanced chemiluminescence substrate (Amersham, Buckinghamshire, U.K.).

γ-Irradiation of cell lines

B lineage cells, 55D1 (61), were irradiated with 6 Gy of ionizing radiation using a Nordion 1.8 kCi ^{137}Cs irradiator as a γ-ray source at 7.8 krad/h. At each time point tested after irradiation, cell viability was determined by trypan blue dye exclusion. The cells were pelleted and resuspended in guanidine isothiocyanate, and RNA was prepared. Cell cycle analysis of irradiated and unirradiated cells was performed by flow cytometry after the cells were stained with Hoechst 33342 dye (62).

Yeast complementation

The mutant *rad51* strain (g896–7Dα) containing the *rad51–1* allele was obtained from John Game (Lawrence Berkeley Lab; Berkeley, CA) (63). Meiotic segregants were obtained from a diploid cross of g896–7Da with w303–1A (*MATa ura3–1 trp1–1 his3–11, 15 ade2–1 leu2–3, 112 can1–100*) by selecting on yeast peptone dextrose supplemented with canavanine (64). A meiotic segregant containing *MATa rad51–1 ura3–1 trp1–1 leu2–3, 112* was used for subsequent studies.

Rabbit *RAD51* cDNA was cloned into the pYES2.0 shuttle vector under the *GAL1* promoter. This vector was introduced by transformation into the yeast *rad51* strain, and

transformants were selected in synthetic media without uracil. *RAD51* expression was induced with 5% galactose. After 2 days, transformant yeast cells were plated on YpGal (5%). For DNA double-strand break repair studies, the plates were exposed to ionizing radiation as described above at 7.8 krad/h and incubated at 30°C. The percentage of viable cells after 3 days, relative to time zero, was determined. The percentage of viable cells after 1 wk was similar to that determined after 3 days. Each sample was determined in duplicate, with typically <10% variation.

For mating type-switch complementation, *rad51 S. cerevisiae* pYES2.0 transformants were additionally transformed with pGHOT-*GAL3* (Trp^+) (65), containing the *HO* endonuclease gene controlled by the *GAL1* promoter. pYES2.0, pGHOT-*GAL3* double transformants were selected by growing them in synthetic media lacking both uracil and tryptophan but containing galactose (5%) to induce switch through expression of *HO* endonuclease. After 40–48 h of induction, double-transformant cells were plated onto synthetic dextrose media without uracil and tryptophan. Individual colonies were tested for switch by mating with both *MAT***a** and *MATα* tester strains. The genotype of the *MAT***a** tester is *his7 ura1 cdc4–1*, whereas the genotype of the *MATα* is *his7 cdc4–1 hom3 can1–100 sap3*. Importantly, because the genetic backgrounds of *rad51* and tester *S. cerevisiae* are heteroallelic at multiple alleles, one can test for switch by genetic cross-complementation. For example, neither *rad51* nor tester haploid strains are able to grow in media lacking histidine; however, if mating has occurred, diploid cells are able to grow on media deficient in histidine. Because *MAT***a** (*rad51–1*) cells can only mate with the *MATα* tester, all unswitched cells will only grow when spotted onto synthetic dextrose plates containing a lawn of the *MATα* tester. Conversely, *rad51* cells that have switched can grow on synthetic dextrose plates containing the *MAT***a** tester. *S. cerevisiae rad51* cells containing rabbit *RAD51* that had switched mating types from *MAT***a** to *MATα* were tested for their ability to switch back to *MAT***a** by mating with the *MATα* tester strain.

Results

Cloning and sequence analysis of rabbit RAD51

We PCR-amplified *RAD51* from rabbit thymus cDNA using oligomers derived from human *RAD51* and determined the nucleotide sequence (Fig. 1A). Genomic *RAD51* was cloned from rabbit genomic phage and plasmid libraries. By restriction mapping and Southern blot analysis, we identified four overlapping $RAD51^+$ phage clones and one nonoverlapping $RAD51^+$ plasmid clone (Fig. 1B). We determined the nucleotide sequence of the fragments that hybridized with *RAD51* and identified 10 *RAD51* exons ranging in size between 60 bp and 123 bp. The exons were separated by as little as 200 bp and as much as 10 kb. To confirm the restriction map, and to localize the nonoverlapping plasmid clone containing exons 1 and 2, we performed Southern blot analysis of *Xba*I-digested genomic DNA using exon-specific probes. The probes for exons 3 to 10 hybridized with *Xba*I fragments identical in size to those cloned in the phage (Fig. 2A). The probe for exons 1 and 2 hybridized with the 9-kb *Xba*I fragment. On the basis of Southern hybridization of *Hin*dIII-digested rabbit genomic DNA with probes for exons 2 and 3 (data not shown), we estimate that this plasmid fragment lies 6 kb upstream of the DNA in phage 11.1 (Fig. 1B). Based on the restriction

map of the phage and plasmid clones, we conclude that the entire *RAD51* gene spans a region >35 kb.

Tissue expression

We examined the expression of *RAD51* in various tissues by poly(A)$^+$ Northern blot analysis and found that it was expressed in nine lymphoid tissues, including sacculus rotundus, Peyer's patches, mesenteric lymph node, small intestine, spleen, bone marrow, omentum, thymus, and appendix (Fig. 2B). *RAD51* expression was undetectable in brain but was found at low levels in lung, liver, and kidney (data not shown).

We detected three different mRNA species, two in mitotic tissues (2.1 kb and 1.9 kb) and a third in testis (1.8 kb) (Fig. 2B). To determine whether all the *RAD51* exons are represented in each mRNA species, we hybridized poly(A)$^+$ Northern blots of appendix and testis RNA with exon-specific probes. We found that, although both the 2.1-kb and 1.9-kb mRNA species hybridized to each exon probe, the 1.8-kb mRNA species from testis did not hybridize to the exon 1–2 probe (data not shown). These data suggest that the 1.8-kb mRNA species lacks the first two exons of *RAD51* and that the 1.9-kb and 2.1-kb mRNA species result from either differential transcription start sites or differential polyadenylation.

Function of RAD51

Recombinational DNA repair.—In *S. cerevisiae*, DNA double-strand breaks, such as those caused by ionizing radiation, are frequently repaired by homologous recombination involving *RAD51* (34, 54, 55). Accordingly, *RAD51* mRNA levels are up-regulated after exposure to DNA-damaging agents (35, 36, 66). In mammals, it also appears that homology-directed repair is an important DNA repair pathway (67). We determined whether rabbit *RAD51* was up-regulated in a manner similar to that of yeast *RAD51* in cell lines in response to irradiation. We irradiated rabbit B cell lines with 6 or 7 Gy of ionizing radiation and measured *RAD51* expression by Northern blot analysis of poly(A)$^+$ RNA (Fig. 3A). By analyzing *RAD51* mRNA levels before and at 1, 3, 4, 6, 12, and 18 h after irradiation, we found that *RAD51* expression increased significantly soon after irradiation. After normalizing *RAD51* mRNA to the amount of β-actin mRNA, we found, in each of four experiments at 6 Gy and two experiments at 7 Gy, a 2- to 15-fold increase in *RAD51* mRNA, with peak induction occurring at 6 h after irradiation (t test = $p < 0.1$; Fig. 3B). We conclude that *RAD51* mRNA is induced in B cell lines after exposure to ionizing radiation, a finding consistent with the potential involvement of *RAD51* in homology-directed DNA double-strand break repair.

Ionizing radiation can cause DNA damage that arrests the cell cycle (68). Because it appears that Rad51 protein is regulated by the cell cycle in mammals (69, 70), we tested whether the increase in *RAD51* mRNA in 55D1 cells after irradiation resulted from cell cycle arrest at a stage when *RAD51* mRNA accumulates. We analyzed the cell cycle profiles of irradiated (6 Gy) and unirradiated cells by staining them with Hoechst 33342 dye and found that the profiles were nearly identical (Fig. 3C). We conclude that the increase in *RAD51* mRNA after exposure to ionizing radiation does not result from cell cycle arrest at a phase where

RAD51 mRNA accumulates but, instead, is likely due to other biological effects resulting from ionizing radiation.

To examine whether rabbit *RAD51* can function in homologous recombination, we tested whether it could complement the homologous recombination defect in *S. cerevisiae rad51* mutants. Because of this defect, *rad51* mutants are hypersensitive to ionizing radiation. We cloned rabbit *RAD51* into a yeast expression vector, introduced this vector into *rad51* mutant yeast, and determined whether the yeast survived exposure to ionizing radiation. As shown in Fig. 4, we found that *rad51* yeast that expressed rabbit *RAD51* were less sensitive to ionizing radiation than were *rad51* mutant yeast. For example, *rad51* cells expressing rabbit *RAD51* that were exposed to 23.4 krad of γ-irradiation were ~10 times more viable than *rad51* cells expressing either vector alone or rabbit *RAD51* in the antisense orientation. These data demonstrate that rabbit *RAD51* can partially complement the γ-ray sensitivity of the *rad51* mutant. Because yeast use homologous recombination to repair DNA damage caused by ionizing radiation, we conclude that rabbit *RAD51* can function in recombinational repair of DNA.

Gene conversion.—Because ionizing radiation primarily stimulates recombinational repair that results in gene conversion (71), we decided to determine whether rabbit *RAD51* can function in sitespecific repair of a DNA double-strand break that results in gene conversion. Yeast mating-type switch occurs by gene conversion, which is initiated by *HO* endonuclease through a site-specific DNA double-strand break at the *MAT* locus. When one of two flanking loci, *HMRa* or *HMLα*, is used as a donor, the recipient *MAT* gene with the double-strand break is converted to the opposite mating type (72). We tested whether rabbit *RAD51* could complement a *rad51* (*MAT***a**) yeast strain that is unable to switch mating types. Cells were tested for their ability to switch by measuring whether they could mate with cells of the original type after induction of switch. For example, *rad51* cells begin as *MAT***a** and can only mate with *MATα* strains. After they switch, these cells become *MATα* and can then mate with *MAT***a** strains. We expressed both rabbit *RAD51* and *HO* endonuclease in *MAT***a** *rad51* cells and assayed for mating type switch to *MATα* by testing whether the switched cells could mate when mixed with a *MAT***a** tester strain. As a positive control, we expressed yeast *RAD51* in *rad51* cells and determined that >20% of the cells undergo mating type switch (Table I). Six of 2851 *rad51* yeast isolates expressing rabbit *RAD51* mated with the *MAT***a** tester strain, indicating that they had switched mating types. In contrast, none of >5000 *rad51* isolates expressing either vector alone or the rabbit gene in the antisense direction grew on *MAT***a**-coated minimal media plates. Statistical analysis using Fisher's exact test showed a significant difference ($p < 0.0015$) in mating type-switch frequencies between *rad51* cells containing rabbit *RAD51* and those containing vector alone and rabbit *RAD51* in the antisense orientation.

To rule out the possibility that the cells that had switched mating type were $RAD51^+$ revertants, two of the six *MATα* isolates containing rabbit *RAD51* were retested for their ability to switch mating type. If $RAD51^+$ revertants were responsible for the observed mating-type switch, then we would expect the frequency of switching to *MAT***a** to be >20%. In both isolates, the frequency of mating type switching remained very low (<5%), indicating that the *rad51–1* allele had not reverted back to wild-type (data not shown). These

data indicate that *rad51* yeast expressing rabbit *RAD51* can switch mating types, suggesting that rabbit *RAD51* functions in gene conversion.

RAD51 expression and ongoing somatic gene conversion

RAD51 mRNA levels during an immune response.—Recently, Winstead and Knight (unpublished data) identified somatic gene conversion-like events within clonally related VDJ sequences isolated from regional popliteal lymph node germinal center lymphocytes after local immunization. To test whether *RAD51* expression is up-regulated in similar circumstances, we immunized rabbits with keyhole limpet hemocyanin and performed Northern blot analysis on poly(A)$^+$ RNA isolated from the regional popliteal lymph nodes (PLN)[4] (Fig. 5). We found a striking increase in *RAD51* expression. By densitometric analysis of six independent experiments, the level of *RAD51* expression in the PLN from the immunized leg was 13-fold ($p < 0.025$) greater than *RAD51* expression in the PLN from the unimmunized leg.

RAD51 mRNA and protein levels in appendix during ontogeny.—We analyzed the expression of *RAD51* in rabbit appendix because somatic gene conversion occurs there starting at ~4 wk of age. Although *RAD51* is expressed in appendix of 2-wk-old animals, much higher levels of both the 2.1-kb and the 1.9-kb *RAD51* mRNAs are evident at 6 wk of age (Fig. 6A). By densitometric analysis, normalizing both mRNA species to β-actin mRNA, we found that in each of four independent experiments, *RAD51* mRNA was 2- to 5-fold higher in 6-wk-old rabbits relative to the level observed in 2-wk-old or adult rabbits (*t* test = $p < 0.1$; Fig. 6B). These data show that *RAD51* expression increases at the same time at which Ig genes undergo somatic gene conversion.

To ascertain whether the increase in *RAD51* mRNA is reflected at the protein level and whether it is found in B lymphocytes, we performed Western blot analysis on FACS-sorted appendix B lymphocytes from rabbits of different ages. We generated antiserum against Rad51 by immunizing rabbits with a His-tagged fusion protein and used this antiserum for Western blot analysis (Fig. 6C). Using densitometric analysis we found that, in each of six experiments, B lymphocytes from appendix of 6-wk-old rabbits had from 2- to 12-fold more Rad51 ($p < 0.005$) than B lymphocytes from 2-wk-old or adult rabbits. These data show that Rad51 protein levels are increased in B lymphocytes from appendix of 6-wk-old rabbits. The timing of this increase correlates with the timing of Ig gene diversification by somatic gene conversion, suggesting that Rad51 may mediate IgH diversification by gene conversion in rabbit.

Discussion

To date, little progress has been made in identifying *trans*-acting factors involved in somatic diversification of Ig genes by either somatic hyperpoint mutation or somatic gene conversion. Because gene conversion occurs via homologous recombination, protein homologues to those involved in homologous recombination pathways in lower eukaryotes

[4]Abbreviations used in this paper: PLN, popliteal lymph nodes; MMS, methyl methanesulfonate.

are ideal candidates for studying somatic gene conversion of Ig genes. One such protein is yeast Rad51, a functional homologue of bacterial RecA. In both yeast and bacteria, these molecules are known to mediate homologous recombination (38, 39, 73–75). In the present study, we demonstrated that expression of a rabbit *RAD51* homologue in a yeast *rad51* mutant restores, in part, DNA double-strand break repair and mating-type switching, both of which occur by homologous recombination. The ability of rabbit *RAD51* to partially restore mating-type switching in the *rad51* strain that has a switch defect demonstrates clearly that rabbit *RAD51* can function in homologous recombination leading to gene conversion. Further, we found that Rad51 levels increase in B lymphocytes from appendix at a time when IgH genes diversify by somatic gene conversion-like events, indicating that Rad51 may mediate IgH gene diversity in rabbits.

Rad51-like molecules have been highly conserved throughout evolution (76), presumably because of their critical role in DNA repair and recombination. Rabbit Rad51 is no exception; it shares a high level of sequence identity with Rad51 homologues from species as distant as *Schizosaccharomyces pombe* (77) and *Xenopus laevis* (78) (Fig. 7). One of the most conserved regions of these genes includes consensus Walker type-A and type-B nucleotide binding domains (79), which are important for *RAD51*-dependent recombination in *S. cerevisiae* (35, 80, 81). Based on the high degree of sequence identity, it seems likely that rabbit Rad51 functions similarly to yeast Rad51.

Yeast *RAD51* is important for repair of DNA double-strand breaks induced by DNA-damaging agents such as γ-irradiation or the radiomimetic drug methyl methanesulfonate (MMS). Repair of DNA double-strand breaks in yeast after damage by these agents occurs by homologous recombination, frequently resulting in gene conversion (82). Experiments from several laboratories have implicated mammalian Rad51 involvement in recombination. Baumann et al. (47) showed that, similar to yeast Rad51, purified human Rad51 pairs homologous DNA molecules and catalyzes strand exchange in vitro. In addition, Xia et al. (83) demonstrated that increased recombination frequencies in immortalized human cell lines correlated with increases in *RAD51* mRNA. Direct evidence that mammalian Rad51 can function in homologous recombination has been obtained through complementation of recombination-deficient yeast. In the present study, we showed that rabbit *RAD51* partially complemented the sensitivity of yeast *rad51* mutants to ionizing radiation. These data are consistent with the studies of Morita et al. (43) and Knight and Barrington (84), which demonstrated that expression of mouse and rabbit *RAD51* in *rad51 S. cerevisiae* could complement sensitivity to methyl methanesulfonate (MMS).

Like yeast, mammalian cells can repair DNA double-strand breaks by homology-directed recombination (67). Consistent with a role in double-strand break repair in mammals, rabbit *RAD51* mRNA is up-regulated in lymphoid cell lines after exposure to ionizing radiation. Although we do not know the exact role of increased levels of *RAD51* mRNA after irradiation, Liu et al. (85) described two CHO cell lines that are mildly sensitive to ionizing radiation, XRCC2 and XRCC3, and showed that they are defective in proteins similar to yeast Rad51. Thus, we suggest that, in rabbit, Rad51 may participate in DNA double-strand break repair of radiation-induced damage. This idea needs to be tested directly.

The inability of rabbit *RAD51* to fully complement the recombination defects in DNA double-strand break repair or mating-type switch in *rad51* yeast could result from its inability to interact optimally with other yeast proteins that are involved in homologous recombination. In yeast, Rad51 interacts with several other *RAD52* epistatic group molecules such as Rad52, Rad54, Rad55, and Rad57 (35, 86–90), and these interactions facilitate Rad51-mediated recombination in vitro (40, 91–95). Milne et al. (86) showed that interactions between Rad51 and other molecules involved in homologous recombination are important in vivo by demonstrating that overexpression of a dominant negative homologue of *Kluyveromyces lactis RAD52* in wild-type *S. cerevisiae* interferes with *RAD51*-mediated repair of damage caused by MMS.

We found that Rad51 levels increase in appendix-derived B lymphocytes of 6-wk-old rabbits, a time at which somatic diversification of IgH genes occurs. Although we suggest that this increase in Rad51 is due to its involvement in gene conversion, we cannot exclude the possibility that the increase results from an increase in cell proliferation. From our data, at least two pieces of evidence suggest that *RAD51* has a role beyond that of cell proliferation. First, *RAD51* mRNA is up-regulated in rabbit cell lines after exposure to ionizing radiation. Because the cell cycle is relatively unaffected by the γ-ray dose used in our studies, the increase in *RAD51* mRNA is not a result of increased proliferation or cell cycle arrest. Second, Rad51 levels in B lymphocytes from appendix of 6-wk-old rabbits are higher than the levels in adult rabbits. By 6 wk of age, the follicular structure of rabbit appendix is fully developed (96, 97), and the high number of germinal centers within the appendix is maintained throughout life as the appendix does not involute in adult rabbits (98). If it is assumed that the percentage of cycling B lymphocytes is similar in these tissues, then the increase in Rad51 is likely to be independent of cell proliferation. It has been difficult to discriminate the role of *RAD51* in recombination in contrast to its role in proliferation because *RAD51* knockout mutations are lethal in vertebrate cells (49, 50). Despite the lethality of *RAD51* mutations in vertebrates, both Tsuzuki et al. (49) and Sonoda et al. (50) showed that *RAD51*$^{-/-}$ cells undergo one to two rounds of cell division before dying, implying that *RAD51* is not essential for proliferation per se. Why do the cells die if *RAD51* is not required for proliferation? Sonoda et al. (50) showed that the *RAD51*$^{-/-}$ cells that did undergo one to two rounds of cell division had increased DNA damage, presumably because DNA damage occurs during replication (99), and *RAD51*-mediated homologous recombination is required to repair this damage. If this model is correct, then any mutation affecting Rad51 recombination activity would also compromise cell viability, making it difficult to separate a role for *RAD51* in homologous recombination from a role in cell proliferation by using traditional mutational analysis. If *RAD51* is involved in somatic diversification by gene conversion, then we would expect to find detectable levels in B lymphocytes within germinal centers in rabbit. In mouse, there are conflicting reports regarding the presence of Rad51 within splenic germinal centers. Li et al. (100) detected *Rad51* in the periarteriolar lymphoid sheath (PALS), but not within germinal centers, consistent with a function of murine *Rad51* or a *Rad51*-like molecule in isotype switching. In contrast, Yamamoto et al. (101) found Rad51 within germinal centers and rarely in the PALS. More detailed analysis of Rad51 levels in germinal center cell subpopulations are needed to resolve this issue.

We also found that *RAD51* mRNA levels increased in the PLN of immunized rabbits. This increase presumably reflects ongoing IgH gene diversification as well as increased proliferation. Rabbits diversify their IgH genes by both somatic gene conversion as well as somatic hyperpointmutation (22–24). Winstead and Knight (unpublished data) recently showed that both of these processes contribute to IgH gene diversification in PLN of immunized rabbits. Although our data do not exclude the involvement of Rad51 in somatic hyperpointmutation of IgH genes, we believe that it is not involved because homologous recombination does not appear to be a major mechanism of hyperpoint mutation (27). Instead, we propose that the increase in *RAD51* mRNA in the PLN of immunized rabbits partially results from increased gene conversion.

In this study, we proposed that *RAD51* is involved in somatic gene conversion of IgH genes in rabbit. Mammalian homologues to other yeast molecules involved in homologous recombination will also likely be involved in IgH gene diversification. For example, another *RAD52* epistasis group member, *RAD54*, has been implicated in somatic gene conversion of chicken IgH genes. Bezzubova et al. (102) showed that a $RAD54^{-/-}$ mutant of an actively gene converting chicken cell line underwent IgH gene conversion at a lower frequency. Genes involved in other DNA repair pathways, such as mismatch repair (103, 104), may also contribute to IgH gene diversity. An understanding of the regulation of DNA repair genes and their pathways within B lymphocytes will help both in elucidating the mechanisms of Ig diversity and in understanding how different species utilize these mechanisms to generate a diverse Ab repertoire.

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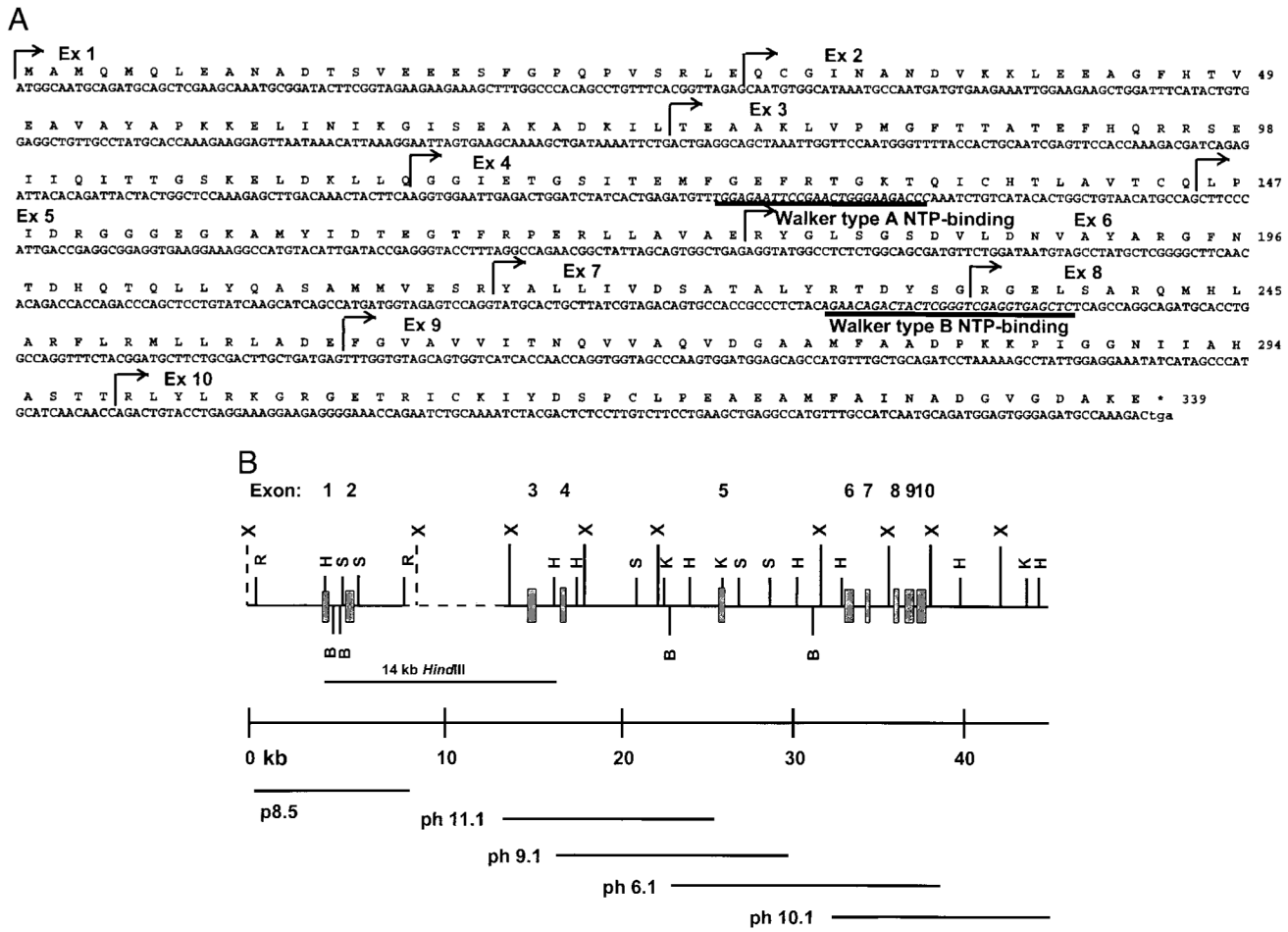

FIGURE 1.

Rabbit *RAD51* cDNA and genomic locus. *A*, Nucleotide and deduced amino acid sequence of rabbit *RAD51* cDNA. Amino acid numbering is shown at the right. Boundaries of exons are marked by arrowed lines. Consensus Walker type-A and type-B nucleotide binding sites are underlined. *B*, Partial restriction map of *RAD51* genomic phage (ph) and plasmid (p) clones. The locations of exons 1–10 (vertical boxes) were determined by restriction mapping and nucleotide sequence analysis. Restriction sites: X, *Xba*I; B, *Bam*HI; H, *Hin*dIII; S, *Sac*II; K, *Kpn*I; R, *Eco*RI (not all *Eco*RI sites are shown). Dashed line indicates region of genomic DNA not cloned. ph11.1 and p8.5 are separated by 6 kb as determined in genomic Southern blot analysis by the hybridization of a 14-kb *Hin*dIII fragment with both exon 2 and exon 3 probes.

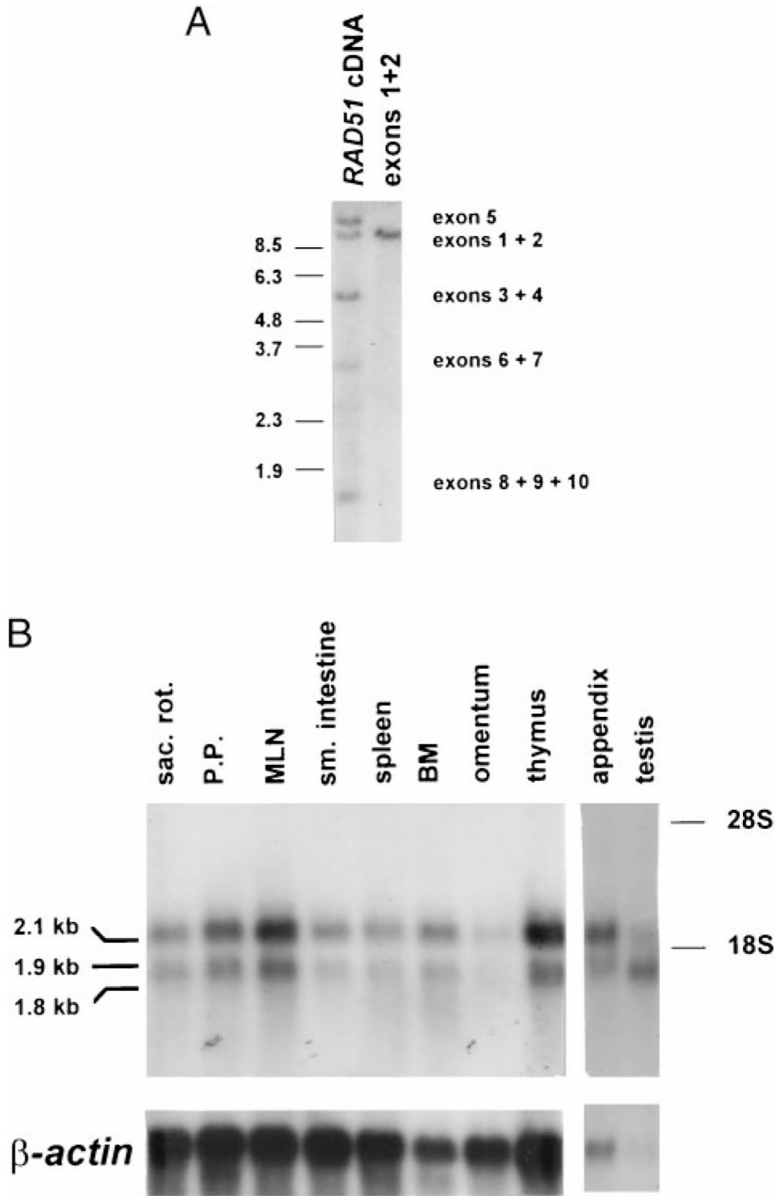

FIGURE 2.

Southern and Northern blot analyses of rabbit *RAD51*. *A*, Southern blot analysis of *Xba*I-digested liver genomic DNA. The blot was probed with a PCR-generated fragment spanning exons 1 and 2 (*right*) and subsequently with full-length *RAD51* cDNA (*left*). Bands corresponding to each *RAD51* exon were similarly confirmed by using exon-specific probes as indicated at the right. The size standard is *Bst*EII-restricted λ phage DNA, and the sizes are indicated on the left. *B*, Northern blot analysis of *RAD51* mRNA expression in selected tissues of 6-wk-old rabbits. Each lane was loaded with 3 μg of poly(A)$^+$ RNA. *RAD51* and β-actin cDNAs were used as probes. Positions of the 28S and 18S ribosomal bands are shown. Sizes of mRNA species are based on *Hin*dIII-digested λ DNA run in formaldehyde buffer.

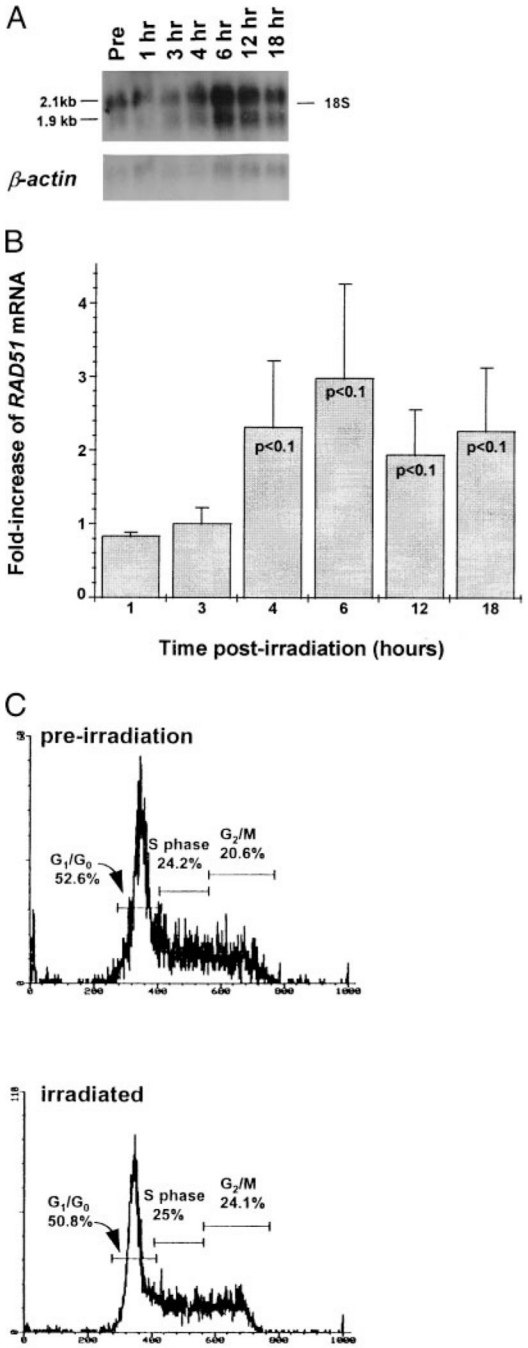

FIGURE 3.

Effects of γ-irradiation on expression of *RAD51* mRNA and on cell cycle of the rabbit cell line, 55D1. *A*, Northern blot analysis of *RAD51* mRNA expression after exposure to 6 Gy of γ-irradiation. Each lane contains 3 μg of poly(A)$^+$ RNA isolated at the given times before and after irradiation. *RAD51* and β-actin cDNAs were used as probes. Position of the 18S ribosomal bands is shown. In this experiment, after normalizing to the amount of β-actin, the level of *RAD51* mRNA at 4 h was 2-fold greater than the level in preirradiated control cells. *B*, Summary graph of four independent experiments measuring *RAD51* mRNA level

following exposure of 55D1 cells to 6 Gy ionizing radiation relative to the *RAD51* mRNA level before irradiation (fold increase). *RAD51* levels for each time point were normalized to the amount of β-actin mRNA by densitometry and the fold increase was determined as the ratio of *RAD51* mRNA level after irradiation to the level before irradiation; p values (t test) for 4, 6, 12, and 18 h time points are given. When data from two additional experiments performed with 7 Gy ionizing radiation are included in the analysis, p values were $p < 0.025$ for the 6 and 12 h time points; $p < 0.05$ for the 18-h time point. *C*, FACS profiles of 55D1 cells before (*top*) and 4 h after (*bottom*) exposure to 6 Gy of γ-irradiation. The cells were stained with Hoechst 33342 dye 1 h before analysis by flow cytometry.

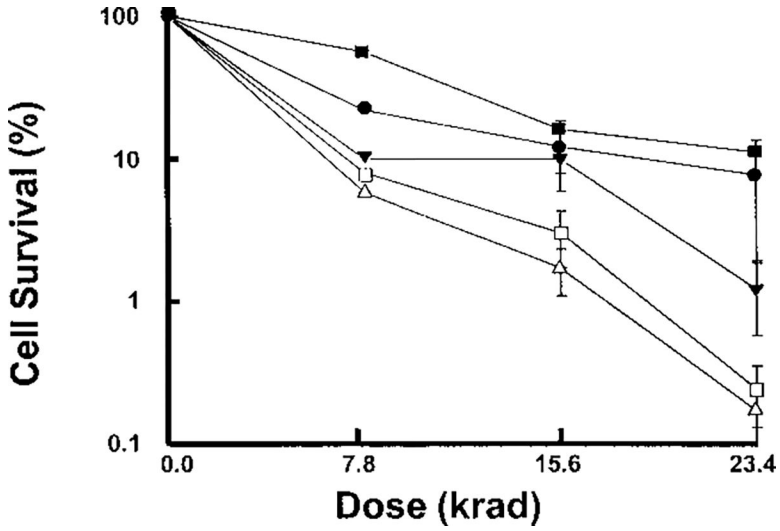

FIGURE 4.

Complementation of *rad51 S. cerevisiae* by rabbit *RAD51* after exposure to γ-irradiation. Fraction of surviving *rad51* yeast containing plasmid with rabbit *RAD51* (▼), rabbit *RAD51* in the antisense orientation (△), vector alone (□), or *S. cerevisiae RAD51* (●) is shown. *RAD51* yeast (■) were also tested.

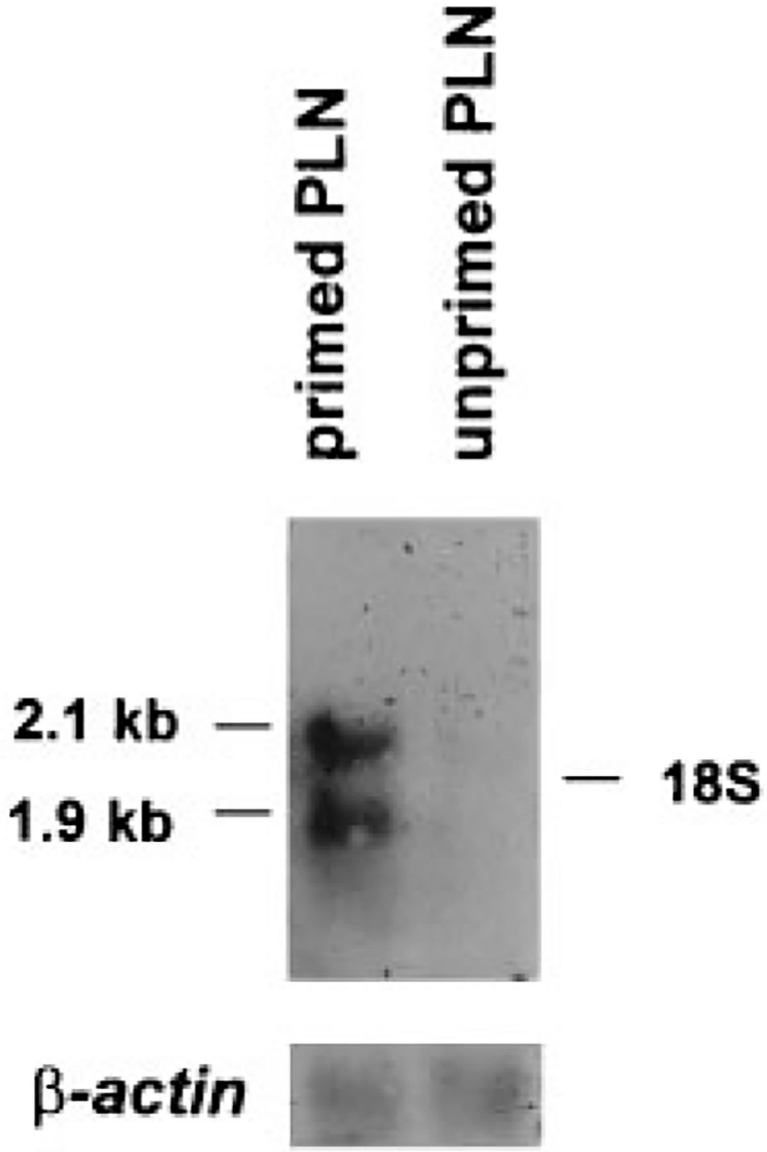

FIGURE 5.

Northern blot analysis of *RAD51* mRNA expression after local immunization. Poly(A)$^+$ RNA (3 μg/lane) was isolated from regional PLN from the immunized leg (primed PLN) and the unimmunized leg (unprimed PLN) of keyhole limpet hemocyanin-immunized rabbits. *RAD51* and β-actin cDNAs were used as probes. Position of the 18S ribosomal bands is shown. After normalizing levels of *RAD51* mRNA to β-actin, the level of *RAD51* mRNA detected in the primed PLN was greater than the level of *RAD51* mRNA in the unprimed PLN ($p < 0.025$ as determined by *t* test).

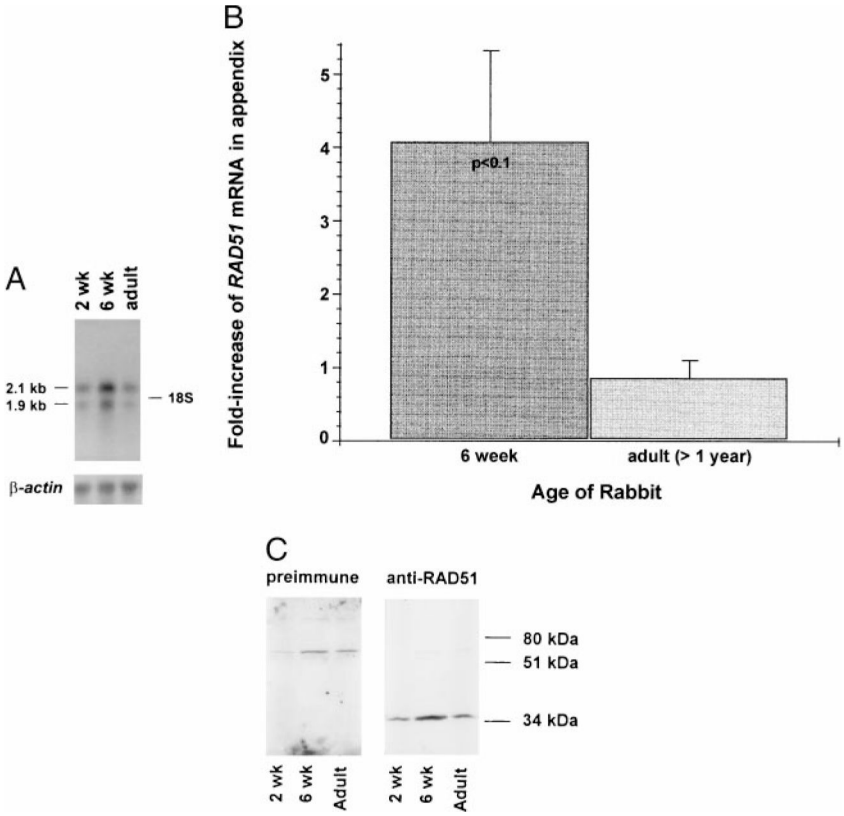

FIGURE 6.

RAD51 mRNA and protein in rabbit appendix. *A*, Northern blot analysis of *RAD51* mRNA expression in appendix from 2-wk-old, 6-wk-old, and adult (>1-yr-old) rabbits. Poly(A)$^+$ RNA (3 μg/lane) from appendices of three different rabbits were pooled for each time point to control for individual animal variation. *RAD51* and β-actin cDNAs were used as probes. Position of the 18S ribosomal bands is shown. In this experiment, the level of *RAD51* mRNA increased 5-fold in appendix from 6-wk-old rabbits relative to that found in 2-wk-old and adult rabbits. *B*, Summary graph of four independent experiments measuring RAD51 mRNA levels in 6-wk-old and adult rabbits relative to that found in 2-wk-old rabbits. The *p* value (*t* test) for the 6-wk-old rabbits is given. *C*, Western blot analysis of Rad51 levels in appendix from 2-wk-old, 6-wk-old, and adult rabbits. Lysates (1×10^6 cells/lane) from FACS-sorted IgM$^+$ appendix cells were immunoblotted with preimmune (*left*) and with anti-human Rad51 (*right*) serum. Positions of the m.w. (kDa) standards are included for comparison. The m.w. of Rad51 is 38 kDa. In this experiment, the level of Rad51 increased 2-fold in appendix from 6-wk-old rabbits relative to that found in 2-wk-old and adult rabbits. Similar results were obtained using IgL$^+$ cells.

FIGURE 7.

Comparison of deduced amino acid sequence of rabbit Rad51 to other Rad51-like molecules. Identity is depicted as dots, and slashes indicate spaces introduced to maximize homology. Walker type-A and type-B nucleotide binding regions are boxed. The N-terminal 52 amino acids from *S. cerevisiae* and the 18 amino acids from *S. pombe* are not included. Ra, rabbit; Hs, *Homo sapien* (accession no. D14134); Mm, *Mus musculus* (accession no. D13473); Ch, chicken (accession no. D09655); Xe, *X. laevis* (accession no. D38489); Dm, *Drosophila melanogaster* (accession no. D17726); Um, *Ustilago maydis* (accession no. L18882); Sc, *S. cerevisiae* (accession no. D10023); Sp, *S. pombe* (accession no. Z22691).

Table I.

Complementation of mating-type switch in rad51 S. cerevisiae

***rad51* Transformant**	**Total Screened**	**Total Switched**[a]	**Switch Frequency (%)**
S. cerevisiae RAD51	886	198	22.3
Rabbit *RAD51* (5′–3′)	2851	6[b]	0.2
Rabbit *RAD51* (3′–5′)	2574	0	0
Vector	2998	0	0

[a] Only patches with more than one papillae were counted.

[b] Statistical analysis using the Fisher's exact test showed a difference between switch in rabbit *RAD51* (5′–3′) and lack of switch in rabbit *RAD51* (3′–5′) and vector ($p < 0.0015$).