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Sequence Conservation of the *rad21 Schizosaccharomyces pombe* DNA Double-Strand Break Repair Gene in Human and Mouse

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The *rad21* gene of *Schizosaccharomyces pombe* is involved in the repair of ionizing radiation-induced DNA double-strand breaks. The isolation of mouse and human putative homologs of *rad21* is reported here. Alignment of the predicted amino acid sequence of Rad21 with the mammalian proteins showed that the similarity was distributed across the length of the proteins, with more highly conserved regions at both termini. The mHR21^{sp} (mouse homolog of Rad21, *S. pombe*) and hHR21^{sp} (human homolog of Rad21, *S. pombe*) predicted proteins were 96% identical, whereas the human and *S. pombe* proteins were 25% identical and 47% similar. RNA blot analysis showed that *mHR21^{sp}* mRNA was abundant in all adult mouse tissues examined, with highest expression in testis and thymus. In addition to a 3.1-kb constitutive mRNA transcript, a 2.2-kb transcript was present at a high level in postmeiotic spermatids, while expression of the 3.1-kb mRNA in testis was confined to the meiotic compartment. *hHR21^{sp}* mRNA was cell cycle regulated in human cells, increasing in late S phase to a peak in G2 phase. The level of *hHR21^{sp}* transcripts was not altered by exposure of normal diploid fibroblasts to 10 Gy ionizing radiation. *In situ* hybridization showed that *mHR21^{sp}* resided on chromosome 15D3, whereas *hHR21^{sp}* localized to the syntenic 8q24 region. Elevated expression of *mHR21^{sp}* in testis and thymus supports a possible role for the *rad21* mammalian homologs in V(D)J and meiotic recombination, respectively. Cell cycle regulation of *rad21,* retained from *S. pombe* to human, is consistent with a conservation of function between *S. pombe* and human *rad21* genes.

INTRODUCTION

A large body of evidence suggests that DNA double-strand breaks (dsbs) are major ionizing radiation (IR)-induced lesions, and their cellular processing may significantly contribute to the fate of the cell after IR exposure (Iliakis, 1991; Kemp *et al.,* 1984; McKay *et al.,* 1994). So-called radiomimetic chemicals and endogenous cellular oxidative processes can also generate DNA dsbs. Physiologically, DNA dsb processing is an integral part of meiosis in germinal cells and also of V(D)J recombination in lymphoid cells. Hence, an ability to restitute DNA dsbs correctly is important to cellular survival in a number of contexts.

Cellular IR exposure can induce progressive genomic instability (Kronenberg, 1994), presumably contributing to the carcinogenicity of IR. IR also induces chromosomal aberrations, a prominent feature of many types of malignancy. DNA dsbs are strongly implicated in the formation of chromosomal aberrations (Naratajan and Obe, 1978; Obe *et al.,* 1992), and it is likely that incorrect or incomplete cellular restitution of DNA dsbs is a significant factor in IR-induced carcinogenesis. Genomic instability is also a feature of a number of human recessive conditions showing increased spontaneous chromosomal instability and a dramatic increase in cancer incidence. These disorders include ataxia telangiectasia, Bloom syndrome, and Fanconi anemia (Sedgwick and Boder, 1991; German, 1993; Liu *et al.,* 1994). Although no human conditions manifesting a clear defect in DNA dsb repair have yet been defined, it is apparent that defective DNA repair is strongly associated with cancer predisposition, as exemplified by diseases such as xeroderma pigmentosum (Cleaver and Kraemer, 1994) and hereditary nonpolyposis coli (Honchel *et al.,* 1995).

Paradoxically, IR is also an effective cancer treatment modality, and approximately half of all cancer patients will receive a course of therapeutic radiation during their illness (McKay and Langlands, 1990). However, the mechanistic basis for the undoubted benefit of this therapeutic modality remains poorly understood. It is possible that the cellular processing of DNA dsbs affects the ultimate outcome of IR exposure to both normal tissue cells (reflected in the incidence of radiotherapy complications) and tumor cells (reflected in the incidence of tumor control by radiotherapy). Greater understanding of the mechanisms of mamma-

Sequence data from this article have been deposited with the GenBank/EMBL Data Libraries under Accession Nos. X98293 and X98294.

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lian cell dsb resolution might therefore hold prospects for improved cancer therapy.

It is apparent that the majority of genes involved in nucleotide excision DNA repair (NER) are strongly conserved in evolution (Hoeijmakers, 1993). Recently, a number of genes involved in DNA dsb repair in yeast were also shown to have structural homologs in mammals (Shinohara *et al.,* 1993; Morita *et al.,* 1993; Muris *et al.,* 1994; Bendixen *et al.,* 1994; Petrini *et al.,* 1995; Kanaar *et al.,* in press). Apart from the structural similarities of these mammalian and yeast genes, partial functional complementation of the yeast mutant phenotype by the mammalian genes has been shown for some but not all of these proteins. Furthermore, structural homologs of DNA repair proteins, first identified in mammals, have been found in yeast. For example, a putative yeast homolog of the Ku70 gene, involved in mammalian DNA dsb rejoining (see Jeggo *et al.,* 1995; and Weaver, 1995, for reviews), was identified in *Saccharomyces cerevisiae* (Feldman and Winnacker, 1993), and recent data indicate that the yeast gene is probably a functional Ku70 homolog (Seide *et al.,* 1996). It is therefore likely that, as for NER, mechanisms for DNA dsb resolution are conserved in mammals.

The protein product of the *rad21* gene of *Schizosaccharomyces pombe* is a nuclear, cell cycle regulated phosphoprotein involved in the repair of IR-induced DNA dsbs and appears to be essential for mitotic growth (Birkenbihl and Subramani, 1992, 1995). The *rad21* gene product may contribute to a DNA damage-dependent recognition or signaling mechanism at the G2 cell cycle checkpoint (Al-Khodairy and Carr, 1992). In an attempt to further the understanding of DNA dsb processing in higher eukaryotes, we isolated and analyzed cDNAs from *Mus musculus* and *Homo sapiens* whose predicted proteins have amino acid similarity to *S. pombe* Rad21.

METHODS

Cloning and nucleotide sequence analysis. Procedures for nucleic acid purification, restriction enzyme digestion, gel electrophoresis, DNA (sub)cloning, PCR, and DNA sequencing were essentially as described (Sambrook *et al.,* 1989). PCR primers used to amplify a portion of the $mHR21^{sp}$ gene were 20-mers, with their 5′ ends at nucleotide 1523 of the final $mHR21^{sp}$ cDNA sequence and nucleotide 38 in the 3′-untranslated region of the final $mHR21^{sp}$ sequence, respectively.

DNA libraries. $mHR21^{sp}$ and $hHR21^{sp}$ cDNA clones were isolated from a Lambda Uni-ZAP mouse testis library (Stratagene) and from a λgt11 human testis library (Clontech), respectively.

Southern blot analysis. Genomic DNA was isolated using proteinase K–phenol/chloroform extraction as described (Sambrook *et al.,* 1989) from the following species: *S. cerevisiae, S. pombe,* garden worm, *Drosophila melanogaster,* wallaby, pig, guinea pig, domestic cat, monkey, and human (placenta). It was digested with *Eco*RI, and 10 μg was size-fractionated on 1% agarose gels, transferred to nylon filters (Bio-Rad) using the manufacturer's protocol, and baked at 80°C. Filters were hybridized with a *rad21* (*S. pombe*) open reading frame (ORF) probe or a $ceHR21^{sp}$ cDNA (ORF) probe. After low-stringency hybridization (56°C) in a solution comprising 7% SDS/0.1 *M* EDTA/0.4 *M* NaPi, filters were washed sequentially at 56°C in 3× SSC/0.1% SDS for 10 min and in 1× SSC/0.1% SDS for 5 min and exposed to radiographic film at −70°C.

Northern blot analysis. Total cellular RNA was extracted from tissues of Balb/c mice or tissue culture cells using the LiCl/urea method (Auffray and Rougeon, 1980). RNA was size-fractionated on 1% denaturing agarose gels, transferred to nylon filters (Bio-Rad) using the manufacturer's protocol, and subsequently baked at 80°C for 1 h or treated in a UV crosslinker (Stratagene). ^{32}P-labeled probes were hybridized to filters at 42°C in 50% formamide-containing mix (Sambrook *et al.,* 1989). After washing (typically, at 65°C for 20–30 min in each of 3× SSC/0.1% SDS and 1× SSC/0.1% SDS), filters were analyzed by phosphorimager (Molecular Dynamics) and/or exposed to radiographic film at −70°C.

Cell culture and cell synchronization. C5RO primary human diploid fibroblasts were routinely cultured in Ham's F10 medium with 15% (v/v) fetal calf serum (FCS), while HeLa cells were cultured in F10:DMEM (1:1) supplemented with 10% FCS, penicillin, and streptomycin. Red-8 murine leukemia cells (Hagemeijer *et al.,* 1982) were maintained in RPMI 1640 medium with 20% FCS. HeLa cells were synchronized using a double thymidine block, as described (Bootsma *et al.,* 1964). At the indicated times after release from the second thymidine block, cells were harvested, and the cell pellets were snap frozen for subsequent RNA isolation. An asynchronous control HeLa cell population, not exposed to thymidine block, was maintained in exponential growth until harvested.

Irradiation experiments. C5RO cells were irradiated in logarithmic growth phase at ambient temperature using a Gammacell 40 cesium-137 source, which supplied a dose rate of approximately 1 Gy/min. After irradiation, cells were cultured at 37°C for the indicated times, when they were harvested by trypsinization and the cell pellets were snap frozen for subsequent RNA isolation.

Chromosomal localization. Fluorescence *in situ* hybridization experiments were performed using biotinylated $hHR21^{sp}$ and $mHR21^{sp}$ cDNA probes, as described (Pinkel *et al.,* 1986; van der Spek *et al.,* 1996). Chromosomes were either banded with diamidinophenylindole (DAPI) and actinomycin D or counterstained with propidium iodide in antifade solution.

Phylogenetic analysis. Using the alignment shown in Fig. 2A, the phylogenetic tree was determined with the PHYLIP software package (Felsenstein, 1993); pairwise distances were calculated with the Dayhoff PAM 001 matrix.

RESULTS

Cloning and Sequence Analysis of Higher Eukaryotic Structural Homologs of S. pombe rad21

The *S. pombe* Rad21 amino acid sequence was used to search the sequence databases using the BLAST algorithm (Altschul *et al.,* 1990). A partially sequenced *Caenorhabditis elegans* cDNA clone (GenBank Accession No. Z14413) with suggestive amino acid sequence homology to Rad21 was identified. The entire sequence of this *C. elegans* cDNA clone was determined, revealing clear additional homology. The predicted gene product had a comparable size to Rad21. In particular, the N-terminus harbored large stretches of identity with Rad21, whereas the remainder of the protein shared a high level of similarity. To test whether the identified *C. elegans* cDNA indeed represented a homolog of the *S. pombe rad21* gene, Southern blot cross-hybridization experiments were performed under low stringency, using the *C. elegans* cDNA to probe a restriction digest of *S. pombe* genomic DNA. As shown in Fig. 1, the *C. elegans* probe indeed hybridized with an *Eco*RI frag-

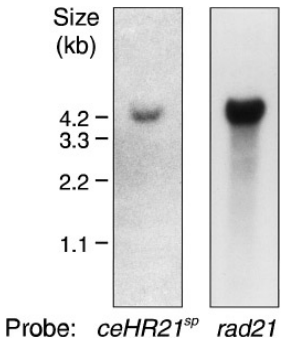

FIG. 1. Southern blot analysis of *Eco*RI-restricted *S. pombe* genomic DNA. (Left) Hybridization with a *ceHR21*^{sp} whole ORF fragment. (Right) The same filter after rehybridization with the *S. pombe rad21* ORF probe.

ment migrating at the same position as the *S. pombe rad21 Eco*RI fragment. Furthermore, the *C. elegans* probe detected a band in *Drosophila* genomic DNA (data not shown), consistent with the idea that the gene is highly conserved. Therefore, the *C. elegans* gene was tentatively named *ceHR21*^{sp}. A subsequent database search using the ceHR21^{sp} predicted amino acid sequence identified an incomplete mouse cDNA with homology to ceHR21^{sp} (Accession No. D37790, DNA database of Japan). The same inquiry also detected the published *S. pombe rad21* gene, confirming the reliability of the hit for the putative *mHR21*^{sp} sequence. PCR primers were designed corresponding to the 3′ region of the ORF in the murine cDNA that had been identified in the database search. An RT-PCR product of the expected size (268 bp) was amplified from mouse testis total cDNA and used as a probe to hybridize a mouse cDNA library. Three cDNAs corresponding to the *mHR21*^{sp} gene were partially sequenced to confirm the sequence obtained from the database and to obtain the missing sequence. The resulting complete mouse gene was designated *mHR21*^{sp}. A cDNA covering the *mHR21*^{sp} ORF was hybridized to a human testis library to obtain human cDNA clones, which were subsequently sequenced. Concomitantly, a number of human expressed sequence tags corresponding to the *hHR21*^{sp} gene were identified in the databases. Recently, a database search using the ceHR21^{sp} predicted amino acid sequence also identified a *C. elegans* genome project clone (Accession No. U40029, submitted to GenBank November 3, 1995). Comparison of this genomic sequence with the *ceHR21*^{sp} cDNA sequence allowed identification of three introns within the *ceHR21*^{sp} genomic sequence (see below), thereby providing information on the genomic organization of the *ceHR21*^{sp} gene.

The Rad21 protein was aligned with its structural homologs from other species (Fig. 2A). The alignment includes an *S. cerevisiae* database sequence, Rhc21p, previously identified as a Rad21 homolog (GenBank Accession No. U23759). The five predicted proteins had start and stop codons in similar locations, with the exception of a small N-terminal extension of the *S. cerevisiae* predicted protein and a small C-terminal extension of the *C. elegans* predicted protein. Homology between the proteins was distributed across the entire ORF region, but there were more highly conserved regions at the N- and C-termini. For example, the human and *S. pombe* proteins shared 59% identity within the N-terminal 92 amino acids (Fig. 2A). In addition, two clusters of basic residues consistent with NLS sequences were conserved (Figs. 2A and 2B), and highly conserved "anchor regions" were dispersed throughout the predicted proteins. When the hHR21^{sp}/mHR21^{sp} pairwise comparison was excluded (as these proteins were nearly identical), the mean value for percentage identity for all nine remaining pairwise comparisons was 25 ± 2% (SE), and for percentage similar amino acids in the same group it was 49 ± 2% (data calculated from Fig. 2A). Features of *S. pombe* Rad21 in comparison with the other species are summarized in Table 1.

The PROSITE protein motif library failed to reveal major structural motifs indicating a particular biological function for these proteins. However, consistent with Rad21 being a phosphoprotein (Birkenbihl and Subramani, 1995, and see below), numerous putative phosphorylation motifs were detected, spread across the length of the predicted proteins. In the mammalian sequence, these included motifs for cyclic-AMP-dependent protein kinase, casein kinase II, protein kinase C, and DNA-dependent protein kinase (DNA-PK). It is currently unknown which of these putative motifs for posttranslational modifications are physiologically significant.

The mHR21^{sp} protein (635 amino acids) was 4 amino acids longer than hHR21^{sp}, with the extra amino acids occurring in a glutamine-rich region of the proteins around the junction of the N-terminal two-thirds and C-terminal one-third (Fig. 2B). Other structural features of the mammalian predicted proteins were also confined to the C-terminal half of the proteins and included a proline-rich region, a region of alternating acidic and basic residues, an (internal) acidic stretch, and two putative nuclear localization signal (NLS) sequences (Fig. 2B). The site corresponding to the only known mutation in the Rad21 group of proteins (I^{67} → T in the Rad21-45 mutant protein; Birkenbihl and Subramani, 1992), marked with an asterisk in Fig. 2B, was within a highly conserved region of these proteins (Fig. 2A).

A phylogenetic analysis of Rad21 and related primary amino acid sequences from other eukaryotes is shown in Fig. 3. The degree of sequence similarity of the various genes is entirely congruent with the evolutionary relationship of the respective organisms. This is in support of the idea that these genes are all derived from the same ancestral gene.

mRNA expression of mHR21^{sp} *in Different Mouse Tissues*

To obtain possible clues to *mHR21*^{sp} function, its mRNA expression was examined in different mouse

tissues (Fig. 4). A 3.1-kb *mHR21^{sp}* mRNA species was easily detected in all tissues, consistent with the constitutive expression found for other DNA repair genes (Friedberg *et al.*, 1995). In comparison to skeletal muscle, the tissue with the lowest expression of those examined, mRNA levels were highest in testis tissue (approximately sevenfold elevated) and thymus (approximately sixfold elevated). Constitutive *mHR21^{sp}* mRNA expression was also elevated in brain and kidney (both approximately threefold). Unlike the other tissues, a clear smaller mRNA transcript (2.2 kb) was also present in testis (Fig. 4). Phosphorimager quantitation showed the ratio of the 2.2-kb transcript to the 3.1-kb transcript in testis tissue to be approximately 1.4:1. In HeLa cells, the *hHR21^{sp}* transcript size was also 3.1 kb (data not shown).

Increased *mHR21^{sp}* mRNA expression in testis tissue is consistent with a possible role for the *mHR21^{sp}* gene in meiotic recombination, which requires DNA dsb processing. Therefore, to examine further whether expression of either the 3.1- or the 2.2-kb testis transcript occurred predominantly in meiotic or postmeiotic testis parenchyma, testis tissues were separated into meiotic and postmeiotic compartments (spermatocytes and spermatids, respectively; Grootegoed *et al.*, 1986). Figure 4 shows that the expression of the predominant 2.2-kb transcript was largely confined to postmeiotic cells. Expression of the 3.1-kb transcript was not detected in the postmeiotic compartment, whereas it was present in the meiotically active spermatocytes.

Cell Cycle Stage-Specific mRNA Expression of hHR21^{sp}

Expression of the *rad21* gene at the mRNA and protein levels was previously shown to vary with *S. pombe* cell cycle stage (Birkenbihl and Subramani, 1995). To determine whether this feature was conserved in the mammalian structural homologs, the expression pattern of *hHR21^{sp}* mRNA was examined at different stages of the mitotic cell cycle on total RNA extracted from HeLa cells, using double thymidine block cell synchronization in late G1 phase (Bootsma *et al.*, 1964). In comparison to the hybridization used to control for total RNA loading, expression of the specific *hHR21^{sp}* mRNA transcript was observed to vary across the different phases, with an increase starting in the latter half of S phase and extending to peak levels in G2 phase (Fig. 5). A control hybridization with an S-phase-specific probe confirmed the cellular synchronization by the thymidine block and that the mammalian *hHR21^{sp}* mRNA started increasing in late S phase.

mRNA Expression of hHR21^{sp} After Ionizing Radiation Exposure

It is not known whether *rad21* mRNA or protein is induced after IR exposure to *S. pombe* cells, although there was no alteration in the phosphorylation pattern of Rad21 protein after a 900-Gy exposure to wildtype *S. pombe* cells or the *rad21-45* mutant (approximately 50 and 1% survival doses, respectively) (Birkenbihl and Subramani, 1995). A number of eukaryotic genes are IR-inducible at the mRNA level (Fornace, 1992; Keyse, 1993). Some of these inducible genes are, like *rad21*, involved in DNA dsb rejoining [e.g., *RAD51* (Basile *et al.*, 1992) and *RAD54* (Cole and Mortimer, 1989)] or are cell cycle regulated [e.g., c-Myc (Sullivan and Willis, 1989) and GADD45 (Papathanasiou *et al.*, 1991)]. The inducibility of *hHR21^{sp}* mRNA was therefore studied in C5RO human diploid fibroblasts after 10 Gy of IR exposure (Fig. 6). In contrast to the known ionizing radiation-inducible GADD45 gene that was used as a control hybridization, there was no increase in *hHR21^{sp}* mRNA after 10 Gy of IR; there was also no *hHR21^{sp}* mRNA induction after 8 h (data not shown).

Chromosomal Localization of Human and Mouse Structural Homologs of rad21

The chromosomal localization of the mammalian *rad21* homologs was determined to see whether these genes were likely to be defective in any known mammalian mutant cell lines with IR sensitivity or a defect in DNA dsb processing. Using fluorescence *in situ* hybridization, *hHR21^{sp}* was determined to reside on chromosome 8q24, whereas the *mHR21^{sp}* gene localized to the 15D3 region (Figs. 7 and 8).

DISCUSSION

The *S. pombe rad21* gene encodes a nuclear phosphoprotein that is involved in the repair of DNA dsbs and promotes cellular survival after IR exposure (Birkenbihl and Subramani, 1992, 1995). Based on studies

FIG. 2. (A) Alignment of the predicted amino acid sequence of *S. pombe* Rad21 with related primary amino acid sequences from other organisms. Sequences shown here are as follows: CEREVIS (Rhc21p of *S. cerevisiae;* Strunnikov and Koshland, unpublished; GenBank Accession No. U23759), POMBE (Rad21 of *S. pombe;* Birkenbihl and Subramani, 1992), ELEGANS, MOUSE, and HUMAN (ceHR21^{sp}, mHR21^{sp}, and hHR21^{sp}, respectively). Numbers represent the amino acid number in the sequence. Letters in black boxes represent identical amino acids in at least two species, whereas letters in gray boxes represent similar (P, A, G, S, T; E, D, N, Q; V, I, L, M; F, W, Y; R, K, H) amino acids. Gaps introduced into the sequences for alignment optimization are shown as dots. The star shows the position of the *rad21-45* mutation in *S. pombe.* Reading from the N-terminus to the C-terminus, underlined sequences represent two putative NLS sequences, and in the mammalian proteins, a glutamine-rich region, a proline stretch, and a highly charged region, respectively (also see text and B). (B) Schematic representation contrasting features of the predicted hHR21^{sp} protein with those of the predicted mHR21^{sp} protein and showing some other major landmarks. There are four additional amino acids present in the mHR21^{sp} protein, all within the glutamine-rich region (see text).

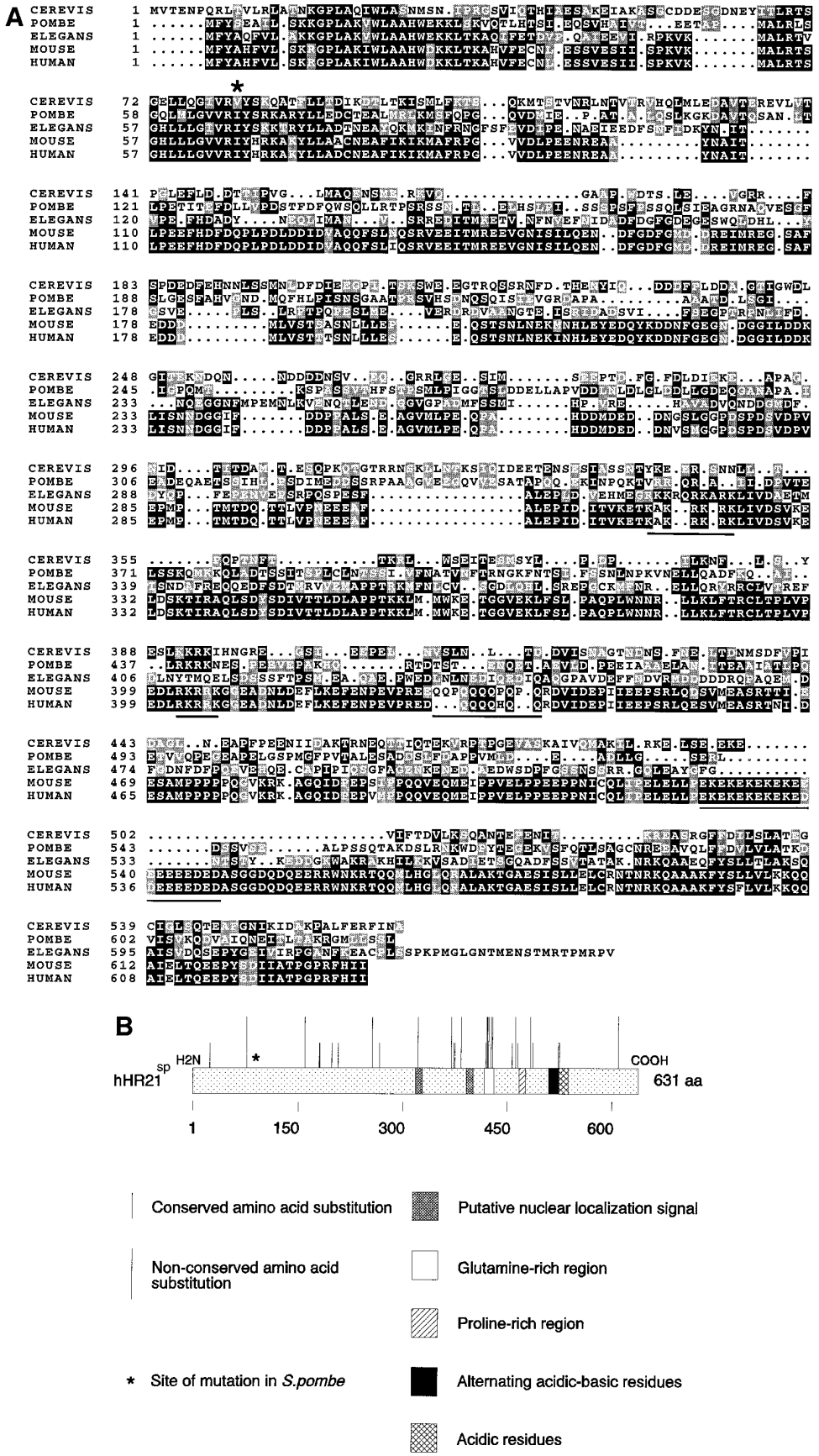
A
CEREVIS
POMBE
ELEGANS
MOUSE
HUMAN
B
sp
H2N
COOH
hHR21
631 aa
1
150
300
450
600
Conserved amino acid substitution
Non-conserved amino acid substitution
* Site of mutation in S.pombe
Putative nuclear localization signal
Glutamine-rich region
Proline-rich region
Alternating acidic-basic residues
Acidic residues

TABLE 1

Features of *rad21* in Comparison to Genes from Other Species whose Predicted Protein Products Bear Amino Acid Sequence Similarity to *rad21*

Gene	Species	Predicted protein (amino acids)	Calculated molecular mass (kDa)	mRNA transcript length (kb)	Introns (bp in genomic sequence)	Mutant	Chromosomal localization (method)	Cell cycle regulated?	References
Rhc21p	*S. cerevisiae*	566	63	Not published	nd	Not published	IVR, near centromere	Unknown	Strunnikov and Koshland, 1995, unpublished (GenBank U23759)
rad21	*S. pombe*	628	68	2.5	One (34–113)	*rad21–45* ($I^{67}\rightarrow T$)	Chromosome III (PFGE/Southern blot analysis)	Yes: peak of mRNA and protein near G1-S transition; peak of protein phosphorylation in G2	Birkenbihl and Subramani (1992, 1995)
ceHR21^{sp}	*C. elegans*	645	73	nd	Three (253–298; 449–496; 1598–1642)	nk	Chromosome III	Unknown	Present
mHR21^{sp}	*M. musculus*	635	72	3.1 (constitutive); 2.2 (Major testis transcript)	nd	nk	15D3 (FISH)	nd	Present
hHR21^{sp}	*H. sapiens*	631	72	3.1 (HeLa)	nd	nk	8q24 (FISH)	Yes: peak of mRNA in late S and G2	Present

Note. The isoelectric point of these proteins is 4.4. nd, not determined; nk, not known.

FIG. 3. Unrooted phylogenetic tree of Rad21 proteins from different species. The five species shown here are the same as in Fig. 2A.

with a *rad21* mutant, it has been suggested that *rad21* may have a regulatory role at the G2 cell cycle checkpoint (Al-Khodairy and Carr, 1992). Here we present the cloning and preliminary analysis of mouse and human homologs of *S. pombe rad21*. Further, a *C. elegans* cDNA corresponding to *rad21* was sequenced, and its sequence was compared with Rad21 and sequences from other species bearing amino acid sequence similarity to Rad21.

A number of lines of evidence indicate that the predicted proteins reported here are very likely to represent true homologs of Rad21. First, the *ceHR21*^{sp} gene recognized a fragment corresponding to *S. pombe rad21* on genomic DNA blots (Fig. 1); apparently, there are no sequences within the *S. pombe* genome more homologous than *rad21* to this *C. elegans* probe. Second, other features of the sequence homology are indicative; the pattern of regions with higher and lower levels of conservation is similar for all homologs. This is even apparent from the comparison of highly similar mouse–human proteins. As shown in Fig. 2A, the N- and C-termini of the proteins display the strongest homology, whereas the middle region is most diverged. The same profile of divergence over the protein is apparent in all of the comparisons. Notably, the highly conserved N-terminus carries the $I^{67}\rightarrow T$ substitution responsible for the *rad21* mutant phenotype. In addition, all homologs have similar sizes, with start and stop codons as well

FIG. 4. Northern blot analysis of *mHR21*^{sp} expression in various adult mouse tissues and testis fractions. Transcripts were detected with a probe containing the whole *mHR21*^{sp} ORF. (Top) Hybridization with the *mHR21*^{sp} probe; (bottom) 18S ribosomal RNA visualized by ethidium bromide staining for RNA loading. Positions of the 28S and 18S ribosomal RNA bands are indicated on the left, while the transcript sizes (in kilobases) are shown on the right.

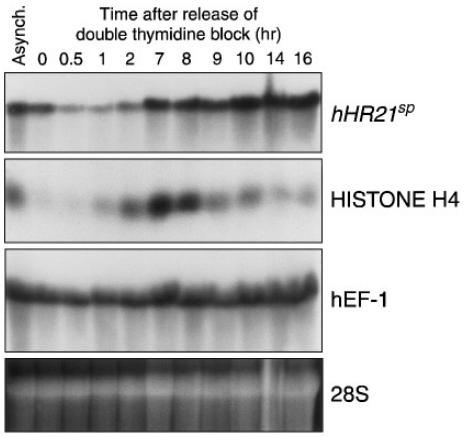

FIG. 5. Northern blot analysis of *hHR21*^{sp} expression according to cell cycle stage in HeLa cells. The pattern in asynchronous control cells is shown on the far left, while the remaining lanes depict patterns in cells 0 to 16 h after release from a double thymidine block (see Materials and Methods). The 0- and 0.5-h timepoints represent late G1, whereas the majority of cells were in S and G2/M phase after approximately 1–8 and 8–16 h, respectively. Control hybridizations were performed with histone H4 as an S phase marker and human elongation factor 1 (hEF-1) as a loading control. The appearance of the 28S ribosomal RNA bands on the corresponding gel is shown in the bottom panel.

as the presumed nuclear localization signals at comparable positions. All have numerous phosphorylation sites. With the exception of the highly charged region near the C-terminus of the mammalian proteins, the hydrophobicity plots of all members are very alike (data not shown). Strikingly, all five proteins have the same, very acidic isoelectric point of 4.4. Furthermore, the overall levels of homology observed are highly significant and are in the same range as that found for many other genes involved in DNA repair (Hoeijmakers, 1993). Third, the phylogenetic relationship of the various Rad21 proteins (Fig. 3) corresponds with the known evolutionary relationship between the respective organisms: *S. pombe* is approximately as far from *S. cerevisiae* as it is from mammals (Huysmans *et al.*, 1983; Sipiczki, 1989). Fourth, the mammalian genes show the same type of cell cycle regulation as the *S. pombe rad21* gene, and the tissue-specific expression profile of the *mHR21*^{sp} gene is as expected for a gene involved in recombination repair. On the basis of the above collective arguments, we conclude that the genes identified here represent descendants of the same ancestral gene. Obviously, this does not exclude the possibility that some species may have more than one copy of the gene due to gene duplication, as has been observed for some other repair genes (Koken *et al.*, 1991; Masutani *et al.*, 1994).

The predicted proteins for all five genes are very acidic. The mammalian products also have an internal acidic stretch in their sequences (Fig. 2B), like that described for nucleolin (Roussel *et al.*, 1992). Although the function of the Rad21 proteins is currently unknown, their acidic nature is consistent with a possible chromatin-binding role. This suggestion is in turn compatible with other features of Rad21, including nuclear localization, an involvement in DNA dsb rejoining, and a possible role in the G2 checkpoint (Birkenbihl and Subramani, 1992, 1995; Al-Khodairy and Carr, 1992). The glutamine-rich region in the C-terminal one-third of the mammalian predicted proteins (Fig. 2B), the only region differing in length between the human and the mouse Rad21, is typical of transactivators (Brinkmann *et al.*, 1995). Similar size variation of monotonic sequences encoding stretches of glutamines have been observed in, for example, the androgen receptor and huntingtin proteins. When exceeding a critical length, these trinucleotide sequences become highly unstable, expanding dramatically. This phenomenon is the cause of disorders such as spinal muscular atrophy and Huntington disease (reviewed in Brinkmann *et al.*, 1995; and Gusella and MacDonald, 1995). A similar mechanism of instability might underlie the size difference between mouse and human Rad21.

Previously, deletion studies identified a probable NLS in the C-terminal one-third of Rad21 (Birkenbihl and Subramani, 1995). In agreement with this observation, the *S. cerevisiae*, mouse, and human polypeptides harbor a putative unipartite NLS at R438 of the *S. pombe* protein (Fig. 2A). However, there is no apparent NLS in ceHR21^{sp} at this site. Interestingly, a possible NLS occurs at another position within the *C. elegans* protein, starting at amino acid R321 of ceHR21^{sp}. This site contains conserved basic residues in all species and may represent a second NLS for the other homologs.

A single amino acid substitution ($I^{67} \rightarrow T$) in the *S. pombe* Rad21 protein is responsible for the mutant phenotype (Birkenbihl and Subramani, 1992). We showed here that this mutation lies in the region most highly conserved between the different species (Figs. 2A and 2B), thereby increasing the likelihood that this mutation defines a functionally relevant region of the pro-

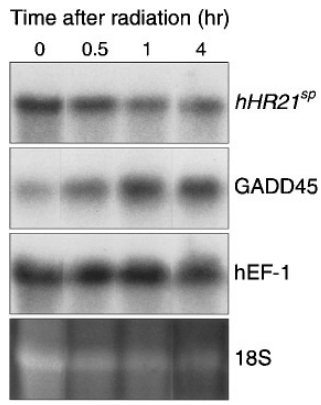

FIG. 6. Expression of *hHR21*^{sp} mRNA after 10 Gy IR exposure to C5RO diploid human fibroblasts, assessed by Northern blot analysis. The same filter was rehybridized with a GADD45 probe as a control mRNA induced by radiation, while human elongation factor 1 (hEF-1) hybridization was used as a control for the amount of RNA loaded in each lane. The bottom panel shows the appearance of the 18S ribosomal RNA band on the corresponding gel.

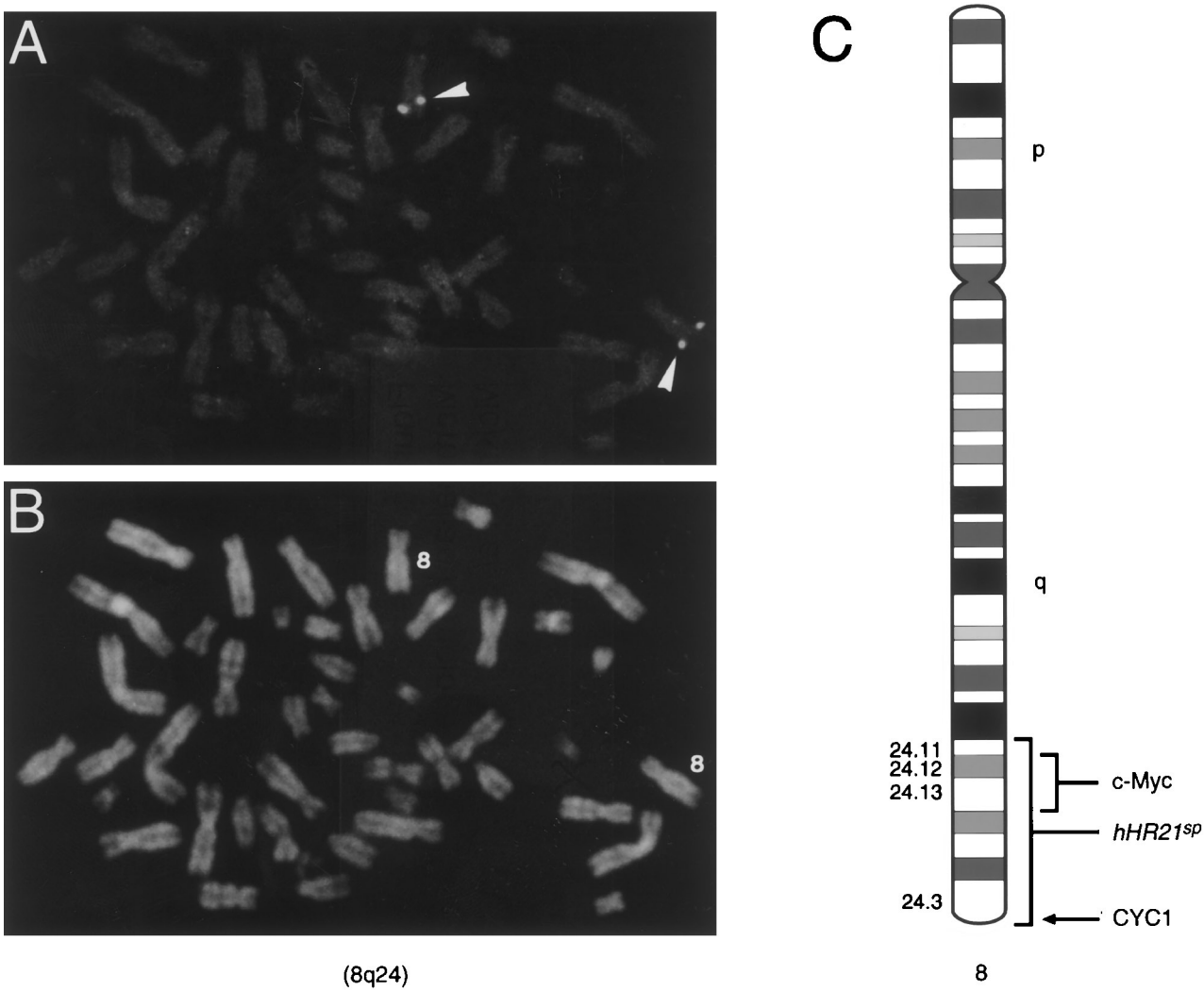

FIG. 7. Chromosomal localization of the *hHR21*^{sp} gene, determined by fluorescence *in situ* hybridization. Biotinylated *hHR21*^{sp} cDNAs were hybridized with human metaphase spreads. (A) Specific double hybridization signals on chromosome 8q24 (arrowheads). (B) DAPI staining used to visualize the chromosomes on the same metaphase spread. (C) Idiogram of chromosome 8. Brackets show the regions within which *hHR21*^{sp} and a reference locus, c-Myc, map. The region between c-Myc and CYC1 is conserved between human and mouse.

teins. Furthermore, although there are many potential phosphorylation sites spread across the Rad21 group of predicted proteins (see Results), the conserved region in which the *rad21-45* mutation occurred also corresponds with the probable region of functionally relevant protein phosphorylation (Birkenbihl and Subramani, 1995).

It has been suggested that the Rad21 phosphoprotein may play a role in the maintenance of the G2 checkpoint (Al-Khodairy and Carr, 1992). In this regard, several kinases in *S. pombe* have recently been identified as having a role in the G2 checkpoint in this organism. These kinases include *chk1* (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994), *rad24*, and *rad25* (Ford *et al.*, 1994). Further, other eukaryotic proteins involved in DNA dsb rejoining have recently been shown to be acting through a pathway that includes DNA-PK (for a review, see Jeggo *et al.*, 1995), and there are potential DNA-PK phosphorylation motifs in the mammalian Rad21 amino acid sequences. Other kinases that are implicated in cellular IR sensitivity and regulate cell cycle events, such as *ATM* and *rad3*^{sp} (see Zakian, 1995), are also candidates for phosphorylation of the Rad21 proteins.

High expression of *mHR21*^{sp} mRNA in thymus and testis compared to that in other adult mouse tissues is consistent with a role for this gene in V(D)J and meiotic recombination, respectively (Fig. 4). Increased steady-state mRNA levels in lymphoid and germ cell tissues have recently been reported for all known mammalian homologs of *S. cerevisiae RAD52* epistasis group genes (Game, 1993) involved in DNA dsb processing: the mammalian equivalents of *RAD51* (Shinohara *et al.*, 1993), *RAD52* (Muris *et al.*, 1994), *MRE11* (Petrini *et al.*, 1995), and *RAD54* (Kanaar *et al.*, in press). In testis, expression of the 3.1-kb transcript appeared to be confined to meiotically active spermatocytic cells (Fig. 4) and thus may play a role in DNA dsb rejoining, a

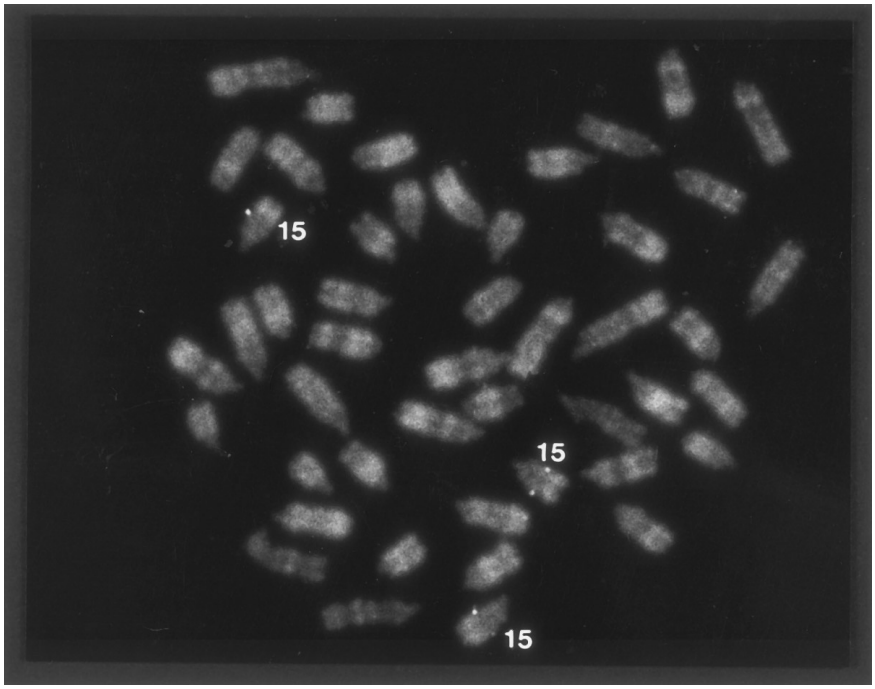

FIG. 8. Chromosomal localization of the *mHR21*^{sp} gene, determined by fluorescence *in situ* hybridization. A biotinylated *mHR21*^{sp} cDNA probe was hybridized with metaphase spreads from the Red-8 murine leukemia cell line, which is trisomic for chromosome 15. Specific hybridization signals were visualized on chromosome 15D3.

DNA transaction necessary in meiosis. In addition to the constitutive 3.1-kb mRNA, a unique 2.2-kb transcript was detected in spermatid cells. It is currently unknown whether the 2.2-kb transcript is biologically active or whether it represents a sterile mRNA species, such as that seen with testis-specific transcripts of some other genes, for example, *c-abl* (Meijer *et al.*, 1987). Examination of mHR21^{sp} protein expression in the different testis compartments may help to resolve this question.

It was previously shown that Rad21 protein and mRNA are cell cycle regulated (Birkenbihl and Subramani, 1995). Such cell cycle regulation is also seen with the human *rad21* gene: *hHR21*^{sp} mRNA increased in late S and was maximal in G2 phase (Fig. 5). This timing is consistent with a role for *hHR21*^{sp} in the G2 checkpoint, as discussed above.

The genomic positions of the human and mouse *rad21* homologs were mapped by *in situ* hybridization to human chromosome 8q24 and mouse chromosome 15D3, respectively (Figs. 7 and 8). c-Myc is also located at these chromosomal sites in both mouse and human, indicating a conservation of chromosomal synteny between mouse and human *rad21* and c-Myc. These chromosomal loci do not correspond with any sites known to be rearranged or deleted in mammalian syndromes that might match the phenotypic features of the available *S. pombe* mutant or with loci known to be involved in laboratory-derived, IR-sensitive mammalian mutants. Further, there are no particular human cancers that have a disproportionately increased rate of loss of this chromosomal region that might suggest involvement of *hHR21*^{sp} in their genesis.

DNA dsb rejoining according to cell cycle stage has not been reported for the sole *S. pombe rad21* mutant. However, the facts that the *rad21* gene is involved in DNA dsb rejoining (Birkenbihl and Subramani, 1992) and that both *S. pombe* and mammalian *rad21* genes are cell cycle regulated (Birkenbihl and Subramani, 1995; and Fig. 5) suggest that the protein product of these genes might participate in cell cycle regulated DNA dsb rejoining. In this regard, the ability of some mammalian IR-sensitive mutants to rejoin IR-induced DNA dsbs has been shown to vary with cell cycle stage. The *xrs* series of mammalian mutants, defective in *XRCC5/Ku80* gene function (see Jeggo *et al.*, 1995), have a DNA dsb repair defect largely confined to the G2 cell cycle stage (Kemp *et al.*, 1984). The *XRCC5/Ku80* gene product forms part of DNA-PK (see Jeggo *et al.*, 1995). Apart from DNA dsb repair, the *XRCC5/Ku80* gene is also involved in V(D)J recombination (see Jeggo *et al.*, 1995), also a cell cycle regulated DNA dsb rejoining mechanism (Schlissel *et al.*, 1993; Lin and Desiderio, 1994). Furthermore, V(D)J recombination occurs in the thymus, where *mHR21*^{sp} mRNA expression is high (Fig. 4). The XR-1 mutant, which, like the *xrs* mutants (Kemp *et al.*, 1984), shows cell cycle phase-specific DNA dsb rejoining (Giaccia *et al.*, 1985), harbors a homozygous deletion of the XRCC4 gene (Li *et al.*, 1995). A human homolog of the *RAD54* DNA dsb repair gene, *hHR54*, is also cell cycle regulated (Kanaar *et al.*, 1996). Taken together, the above findings raise

the possibility that the mammalian *rad21* homologs might participate in the same or similar DNA dsb rejoining pathway(s) as these other cell cycle regulated DNA dsb repair genes.

The study of genes involved in the cellular processing of IR-induced DNA dsbs has significant implications for human health. These include a greater understanding of the mechanisms underlying genomic stability that presumably guard against radiation carcinogenesis and a potential contribution to the management of individuals with cancer. If one or a few genes prove to be involved in the majority of individuals with enhanced normal tissue reactions to radiation therapy, this could open the way for novel preradiotherapy genetic screening, thereby allowing tailoring of radiation therapy schedules to different individuals.

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Note added in proof. Subsequent to the work described in this paper we determined that there is an additional family of genes with amino acid sequence homology to *S. pombe* Rad21. We cloned, sequenced and characterized the human and mouse second homologs.

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