The *rad18* Gene of *Schizosaccharomyces pombe* Defines a New Subgroup of the SMC Superfamily Involved in DNA Repair

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The *rad18* mutant of *Schizosaccharomyces pombe* is very sensitive to killing by both UV and γ radiation. We have cloned and sequenced the *rad18* gene and isolated and sequenced its homolog from *Saccharomyces cerevisiae*, designated *RHC18*. The predicted Rad18 protein has all the structural properties characteristic of the SMC family of proteins, suggesting a motor function—the first implicated in DNA repair. Gene deletion shows that both *rad18* and *RHC18* are essential for proliferation. Genetic and biochemical analyses suggest that the product of the *rad18* gene acts in a DNA repair pathway for removal of UV-induced DNA damage that is distinct from classical nucleotide excision repair. This second repair pathway involves the products of the *rhp51* gene (the homolog of the *RAD51* gene of *S. cerevisiae*) and the *rad2* gene.

Cells of all organisms have evolved an intricate series of DNA repair pathways to counteract the deleterious effects of all types of DNA damage. In *Escherichia coli*, nucleotide excision repair (NER) of UV damage requires the products of six genes. A complex of the UvrA and UvrB proteins binds to DNA and translocates to the site of the damage. The UvrC product then attaches to the complex, displacing UvrA, and the damaged DNA strand is nicked on both sides of the damaged site. The helicase activity of the UvrD product releases the oligonucleotide containing the damage, and DNA polymerase I and ligase complete the repair process (24). In eukaryotes, NER requires considerably more gene products, most of which are highly conserved (20). In *Saccharomyces cerevisiae*, the products of the *RAD1, -2, -3, -4, -10, -14,* and *-25* genes are absolutely required for excision repair of UV damage, whereas there is only a partial requirement for *RAD7, -16,* and *-23*. There is evidence that the products of many of these genes form a multisubunit complex (e.g., see reference 53). In *Schizosaccharomyces pombe*, genes encoding highly homologous proteins have been identified (9, 10, 30, 39), demonstrating the conservation of the classical NER pathway in this yeast.

Null mutations in the *S. cerevisiae* NER genes *RAD1, -2, -3, -10,* and *-14* result in a total deficiency in excision repair of UV-induced cyclobutane dimers and 6-4 photoproducts (28). Null mutants of the *S. pombe* homologs of *RAD1* and *RAD2* (*rad16* and *rad13*, respectively), while showing many properties expected of excision repair-deficient mutants, are still able to excise UV-induced cyclobutane dimers and 6-4 photoproducts at a significant rate (7, 27). These results suggest that, in contrast to *S. cerevisiae*, there is a second pathway in *S. pombe* for removal of UV photoproducts.

Most of the *rad* mutants of *S. pombe* are sensitive to both UV and γ irradiation. Some of these are involved in check-

point control of the cell cycle to radiation (2, 3, 41). Others, which are particularly sensitive to ionizing radiation, are deficient in recombination repair (6, 29, 33, 54). No mutant which is sensitive to ionizing but not to UV irradiation has yet been identified. The *S. pombe rad18-X* mutant (32) is sensitive to the lethal effects of ionizing radiation. It is also sensitive to UV irradiation, and we present evidence in this paper suggesting that the Rad18 protein might be involved in the second pathway for removal of UV damage. We demonstrate that the *rad18* gene is essential for proliferation, and we show that the encoded product is related structurally to the SMC family, all previously described members of which are involved in modulating chromosome structure in mitosis. We have also cloned a homolog of *rad18* from *S. cerevisiae* demonstrating that this gene is evolutionarily conserved.

MATERIALS AND METHODS

Cell strains. All cell strains were isogenic. Crosses were carried out by standard procedures (14).

Cell survival. For UV irradiation, logarithmically growing cells were plated onto yeast extract and irradiated at 254 nm either with a Stratalinker (Stratagene) or with a bank of Philips TUV germicidal lamps. For γ irradiation, cells were irradiated in suspension in yeast extract (14) with a ^{137}Cs source (dose rate 12.8 Gy/min) prior to plating onto yeast extract.

Excision repair. The repair of cyclobutane dimers and 6-4 photoproducts after irradiation with a setting of 200 J m^{-2} from the Stratalinker UV source was measured by using the dot blot/immunochemical procedure described by McCready and Cox (28) and subsequently modified for *S. pombe* (27).

UV endonuclease activity. We used the procedure of Sidik et al. (48) to measure the ability of extracts to incise UV-irradiated DNA. *S. pombe* cells (300 ml) grown to stationary phase were harvested and disrupted by using glass beads in a Microdismembrator (Braun). The extracts in a volume of 4.5 ml were centrifuged at 100,000 × g and dialyzed as described by Sidik et al. (48) to give a final concentration of 5 to 10 mg/ml of protein. For reactions, approximately 100 ng of a 4.5-kb plasmid DNA which had previously been irradiated at a reading of 200 J m^{-2} on the Stratalinker were incubated in 25 μl with 0 to 30 μg of protein extract for 1 h at 37°C. The reaction products were treated as described by Sidik et al. (48).

Cloning and analysis of the *rad18* gene and its *S. cerevisiae* homolog. The *rad18* gene was cloned by complementation of the UV sensitivity of the *rad18* mutant using a *S. pombe* genomic library constructed in the *S. pombe* shuttle vector pUR19 (5) as described in our earlier work (9, 10, 12). DNA was extracted from a complementing clone and used to transform DH5αF' by using standard procedures. The insert was sequenced by using a combination of exonuclease deletions (17) and sequence-specific primers with the dideoxynucleotide procedure and Sequenase 2.

For isolation of the complete *S. cerevisiae* homolog, hybridization probes were

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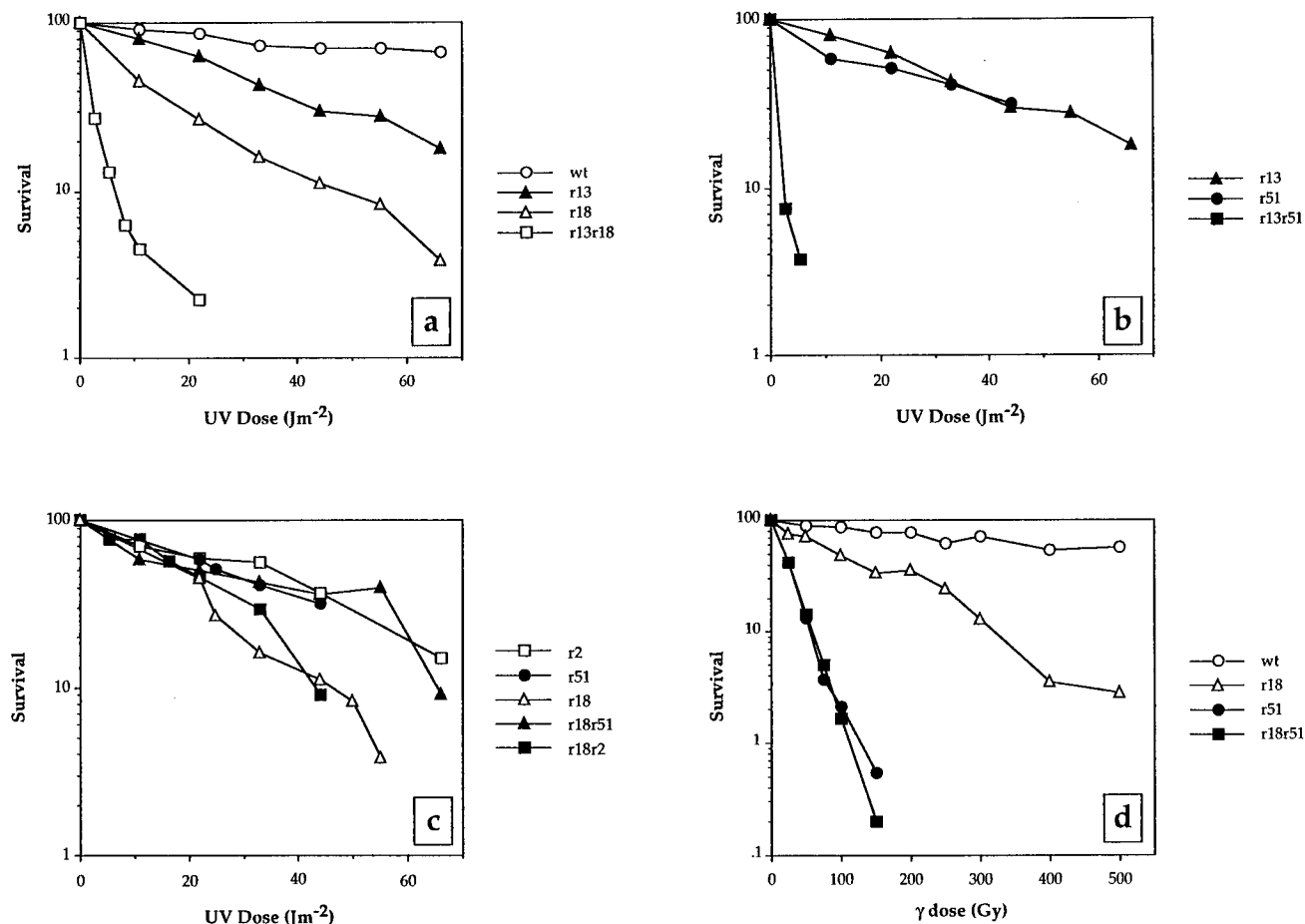
FIG. 1. Radiation sensitivities of single and double mutants. (a to c) Cells (1,000) were plated and irradiated from a low-pressure UV-C lamp at a dose rate of approximately 10 J m^{-2} min^{-1}. (d) Cells in suspension were exposed to different doses of γ irradiation and then 1,000 cells were plated. r13, *rad13*Δ; r18, *rad18-X*; r51, *rhp51*Δ; r2, *rad2*Δ. wt, wild type.

used with an *S. cerevisiae* genome library constructed in pUR18 (5). Inserts in hybridizing clones were restriction mapped and appropriate clones were sequenced by using primer walking.

Molecular biological techniques were as described by Sambrook et al. (45).

Nucleotide sequence accession numbers. The *S. pombe rad18* and *S. cerevisiae RHC18* sequences have been deposited in the EMBL database under accession numbers X80929 and X80930, respectively.

RESULTS

Survival of *rad18* after irradiation. The *rad18-X* mutant is sensitive to both UV and γ irradiation. Its sensitivity following exposure to increasing doses of UV irradiation is greater than that of classical NER-defective mutants such as a *rad13*Δ mutant (Fig. 1a). The sensitivity to increasing doses of γ rays is substantial, but considerably less than that of the exquisitely sensitive *rhp51*Δ recombination-repair mutant (Fig. 1d).

Epistasis analysis. In order to determine in which pathway the Rad18 product functions, we have constructed double mutants of all combinations of *rad18*, *rad13*, *rhp51*, and *rad2* mutants and measured their survival after UV and γ irradiation. *rad13* is the *S. pombe* homolog of *S. cerevisiae RAD2*, a classical NER gene. *rhp51* (21, 29, 46) is the *S. pombe* homolog of *S. cerevisiae RAD51* which is thought to be involved in homologous pairing during recombination (52). Mammalian homologs of *S. pombe rad2*, and by implication *rad2* itself, encode a structure-specific nuclease involved in DNA replica-

tion and/or repair (16, 31, 38; see Discussion). The responses to UV of the double mutants fell into two clearly different categories. Double mutants of *rad13*Δ with either *rad18-X* (Fig. 1a), *rhp51*Δ (Fig. 1b), or *rad2*Δ (31) showed a synergistic response. They were much more sensitive than the single mutants, suggesting that Rad18, Rhp51 and Rad2 operate in a repair pathway or pathways independent of classical NER. In contrast, neither of the double mutants *rhp51*Δ *rad18-X* nor *rad2*Δ *rad18-X* was more sensitive than the single mutants (Fig. 1c). (*rhp51*Δ *rad2*Δ was not viable [31].) Although other explanations have not been ruled out, the simplest interpretation of these data is that Rad18, Rhp51, and Rad2 are involved in the same repair pathway.

With γ radiation, the *rad18-X rhp51*Δ double mutant had a response very similar to that of the *rhp51*Δ single mutant. Both of these were considerably more sensitive than the *rad18-X* single mutant (Fig. 1d). Again this is consistent with Rad18 acting in the same repair pathway as Rhp51 in the response to ionizing radiation.

Removal of photoproducts. In *S. cerevisiae*, the *RAD1*, *-2*, and *-3* genes are absolutely required for excision repair (28). We showed previously that mutations in the homologous *S. pombe* genes *rad16*, *-13*, and *-15* result in only a small reduction in the rate of elimination of UV photoproducts (27). Figure 2a shows that the rates of removal of cyclobutane dimers and 6-4
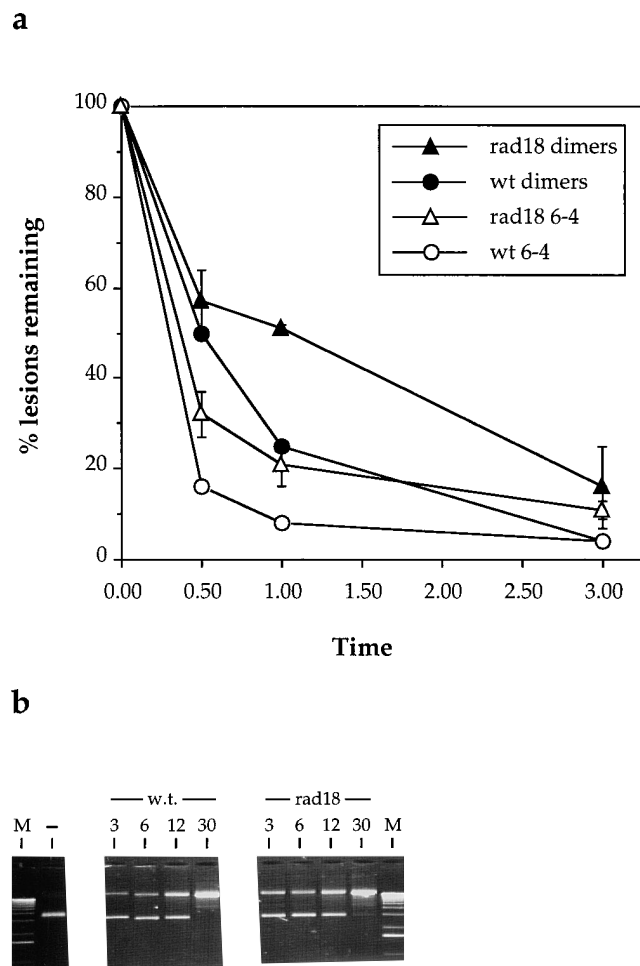
a

b

FIG. 2. Repair of UV damage. (a) Rate of removal of cyclobutane dimers and 6-4 photoproducts in wild-type (wt) and *rad18* cells, using dot blot immunoassay (27). (b) UV endonuclease activity of wild-type and *rad18* extracts. Increasing concentrations of extract convert supercoiled to nicked circles. M, marker lanes (1-kb ladder from Gibco/BRL); −, no extract; amounts of protein (in micrograms) above lanes. The differences in mobility between the DNA in the − lane and the lower band in the + extract lanes result from topoisomerase activity (48).

photoproducts, compared with those for wild-type cells, were likewise reduced in the *rad18* mutant.

Sidik et al. (48) have described an endonuclease activity in *S. pombe* extracts specific for UV-treated DNA. Using an assay which measures this nuclease activity by detecting the conversion of UV-irradiated supercoiled DNA into nicked circles, we have measured the endonuclease activity in extracts from normal and *rad18* cells. With increasing amounts of extract, there was a progressive depletion of supercoiled DNA with corresponding increase in nicked circles. There was no significant difference between activity in wild-type and *rad18* extracts (Fig. 2b). Approximately 60% of supercoiled circles were converted to nicked circles with 12 μg of protein extract and 100% with 30 μg. Similar results were found with *rad13Δ* extracts (not shown). There was negligible activity with any extract on un-irradiated DNA under the same conditions (not shown).

Cloning of the *rad18* gene. Epistasis analysis and the measurement of UV photoproduct repair suggest that Rad18 might act in a novel repair pathway responsible for removal of UV-induced photoproducts and possibly plays a role in recom-

bination repair of double strand breaks. In order to learn more about the nature of Rad18, we have cloned the *rad18* gene by complementation of the UV sensitivity of the *rad18* mutant. Following transformation with an *S. pombe* genomic library and selection for UV resistance, two independent clones were isolated and the DNA from these clones was used to transform *E. coli.* A single plasmid was recovered from each resistant clone. Restriction mapping revealed that the 5-kb insert in the smaller clone, A, was entirely contained within the 7.8-kb insert in the larger clone, F (Fig. 3a).

In order to confirm that the DNA in these plasmids contained the *rad18* gene, the plasmid with the larger insert was transformed into the *rad18* mutant and stable integrants were derived from the transformants. Three separate integrants (which all showed wild-type levels of UV resistance) were crossed with wild-type *S. pombe* and induced to sporulate. No UV-sensitive cells were identified from 500 spores of the three crosses, indicating that the *rad18* plasmid had integrated at the same locus as the *rad18* mutation.

The smaller insert was sequenced in both directions. The sequence (Fig. 4a) revealed a large open reading frame (ORF) of 3.3 kb and two small ORFs, one upstream and one downstream of the large ORF. The short downstream ORF is identical to the *S. pombe rpgL29* gene coding for ribosomal protein L29 (EMBL accession no. X57207) and is highly homologous to ribosomal protein 127a from several organisms. In order to determine which region of the plasmid corresponded to the *rad18* gene, the remaining two ORFs were separated from the ribosomal gene by excising a 4.6-kb *Eae*I-*Pst*I fragment containing the whole of the putative *rad18* gene from the *rad18* clone F (Fig. 3a). This fragment was then cloned into *Not*I-*Pst*I-digested pUR18N (5), and the resulting plasmid was used to transform the *rad18* mutant. The UV and γ survivals of these transformants were compared with those of wild-type strains and strains transformed with the vector alone. The survival of the *rad18* transformants was greatly increased: with UV irradiation the survival was similar to that in wild-type cells (Fig. 5a), whereas with γ irradiation it was slightly lower (Fig. 5b).

The two remaining ORFs appeared to correspond to a single gene as putative splicing signals were present which could result in a splice event creating a single 1,140-amino-acid ORF (Fig. 3a and 4a). In order to confirm the use of this putative intron, *rad18* cDNA was analyzed by PCR and sequencing. Primers AM12 and AM13 flanking the putative intron (Fig. 3a) were used in PCR with either *S. pombe* genomic DNA or an *S. pombe* cDNA library as template. PCR products were produced with both templates, that from the cDNA (ca. 400 bp) being about 100 bp shorter than that from genomic DNA (ca. 500 bp). The sequencing of the product derived from cDNA confirmed the presence of the intron indicated in Fig. 3 and 4.

Since the beginning of the smaller *rad18* insert was only about 200 bp upstream from the putative ATG start codon of the *rad18* gene, a further 900 bp upstream of the ORF was sequenced using a 1.2-kb *Eco*RI-*Pst*I fragment derived from the larger insert. No other ORFs were revealed in this upstream region. This suggested that the two identified ORFs defined the translated region of the *rad18* gene. This was further supported by the construction of a plasmid containing the putative full-length cDNA (including only the two exons) which rescued the UV sensitivity of the *rad18* mutant to approximately wild-type levels.

Northern analysis of RNA. Northern analysis using the 0.75-kb *Eco*RI fragment (Fig. 3a) as probe revealed a transcript of about 3.5 kb, consistent with the size of the *rad18* gene. We have measured the level of the *rad18* transcript
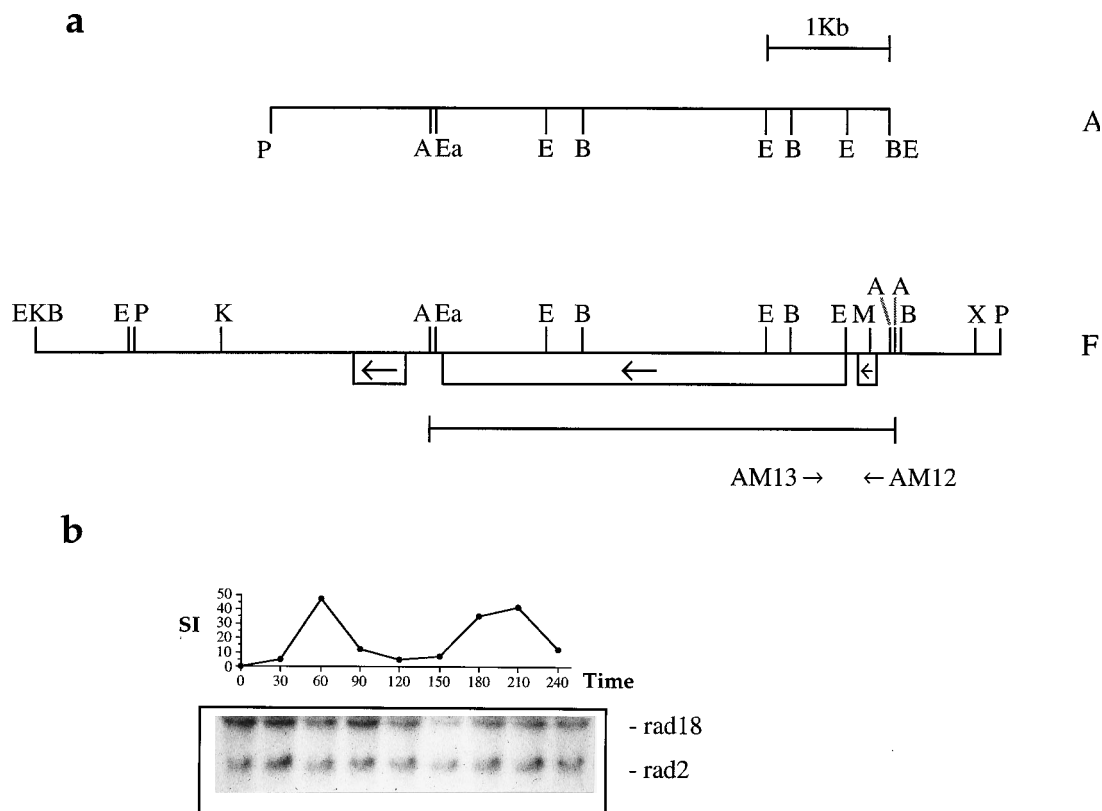
a

1Kb

P   A Ea   E   B   E B   E   BE   A

EKB   E P   K   A Ea   E B   E B   E M A A B   X P   F

AM13 →   ← AM12

b

FIG. 3. Restriction map and expression of the *rad18* gene. (a) Restriction maps of inserts in *rad18*-complementing plasmids. A, *Acc*I; B, *Bam*HI; E, *Eco*RI; Ea, *Eae*I; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; X, *Xba*I. Boxes with arrows represent ORFs. The bar below the diagram shows the *Acc*I fragment removed for the gene disruption. AM12 and AM13 are PCR primers used to confirm the presence of the intron. (b) Northern blot of *rad18* gene. RNA was extracted from selection synchronized *S. pombe* cells at various times. Maximum septation index (SI) occurred at 60 and 210 min. RNA samples were electrophoresed in glyoxal gels, transferred, and hybridized with the 0.75-kb *Eco*RI fragment from the *rad18* gene (see panel a), together with a *rad2* gene probe as control. Differences in intensity are attributable to loading differences.

through the cell cycle in cells synchronized by centrifugation through a 7 to 30% lactose gradient (4). The septation index peaked at 60 and 210 min after inoculation of the synchronized cultures. The level of the *rad18* transcript did not change significantly through the cell cycle (Fig. 3b). We also measured the *rad2* transcript levels, which, like the *rad18* mRNA, did not change significantly.

S. cerevisiae homolog of rad18. Comparison of the *rad18*-encoded sequence with other sequences in the GenBank, EMBL, and cDNA databases by using BLAST and TFASTA alignment programs revealed a homologous sequence from *S. cerevisiae* in a truncated ORF in the region upstream of the *NAM2* mitochondrial leucine tRNA synthetase gene (23). This ORF, which appeared to encode an *S. cerevisiae* homolog of Rad18, reads in the opposite orientation from the *NAM2* gene and only the first 500 or so nucleotides had been sequenced. In order to isolate the complete *S. cerevisiae* homolog, we first amplified a product from *S. cerevisiae* DNA by PCR using primers from the ends of the published sequence upstream of *NAM2*. This PCR product was used as a probe to hybridize against a *S. cerevisiae* genomic library. The first clone obtained contained a 1.8-kb insert with 800 bp of sequence downstream of the previously sequenced DNA. With a probe from the C-terminal end of this sequence a second hybridization with the library produced a plasmid with a 5.5-kb insert. Restriction mapping and sequencing revealed that this insert contained the complete *S. cerevisiae* gene homologous to *rad18*. We have

designated this gene *RHC18* (Rad homolog in *cerevisiae*). *RHC18* is identical to ORF L3502.2 from the chromosome XII sequencing project (GenBank accession no. U19104).

Computer analysis of Rad18 and Rhc18. The predicted products of the *rad18* and *RHC18* genes are proteins with 1,140 (131 kDa) and 1,114 (128 kDa) amino acids (aa), respectively. The predicted isoelectric points of both are 7.4. Alignment (Fig. 6) shows an overall identity of 25%, with several regions of high sequence similarity, especially around the nucleotide binding site (see below) (50% identity over 100 aa) and at the C terminus (48% identity over 87 aa).

In order to investigate the structural organization of the Rad18 and Rhc18 products in more detail, they were analyzed for coiled-coil regions by Jeff Stock (Princeton University) using the New Coil program (26). This analysis showed that Rad18 and Rhc18 have very similar and extensive regions of coiled coils (Fig. 7). It also demonstrates that these proteins are closely related to the recently described SMC family (51) containing the *S. cerevisiae* Smc1 and Smc2 proteins and various other proteins of yeast and mammalian origin involved in chromosome structure and segregation (see Discussion). The proteins in this family have the following characteristics (see references 13 and 19 for reviews): they are all 1,100 to 1,500 aa in length. They have a Walker type A nucleotide binding site at about aa 40 or aa 120, two-coiled coil regions, the first of 240 to 350 aa separated by a spacer of 160 to 210 aa from the second region of about 280 to 380 aa. There is also a highly

a

[DNA sequence and predicted amino acid sequence blocks — microtext, with residue position numbers in the right margin running to 6, 32, 47, 72, 98, 123, 148, 174, 199, 224, 250, 275, 300, 326, 351, 376, 402, 427, 452, 478, 503, 528, 554, 579, 604, 630 (left panel) and 655, 680, 706, 731, 756, 782, 807, 832, 858, 883, 908, 934, 959, 984, 1010, 1035, 1060, 1086, 1111, 1136, 1140, then 18, 43, 68, 94, 119, 144, 148 (right panel). Boldface annotations include CKII, NLS, Nucleotide binding.]

b

Rad18

NLS   ATP binding site   Coiled Coils   Conserved Domain

Rhc18

FIG. 4. DNA sequence of the *rad18* gene and amino acid sequence of its predicted protein product. (a) The coding sequence is in uppercase; the noncoding sequence is in lowercase. In boldface type are shown the casein kinase II site (CKII), the nuclear localization signals (NLS), the nucleotide binding site, and the coiled-coil regions. (b) Summary of the important features of the encoded products of the Rad18 and Rhc18 proteins.
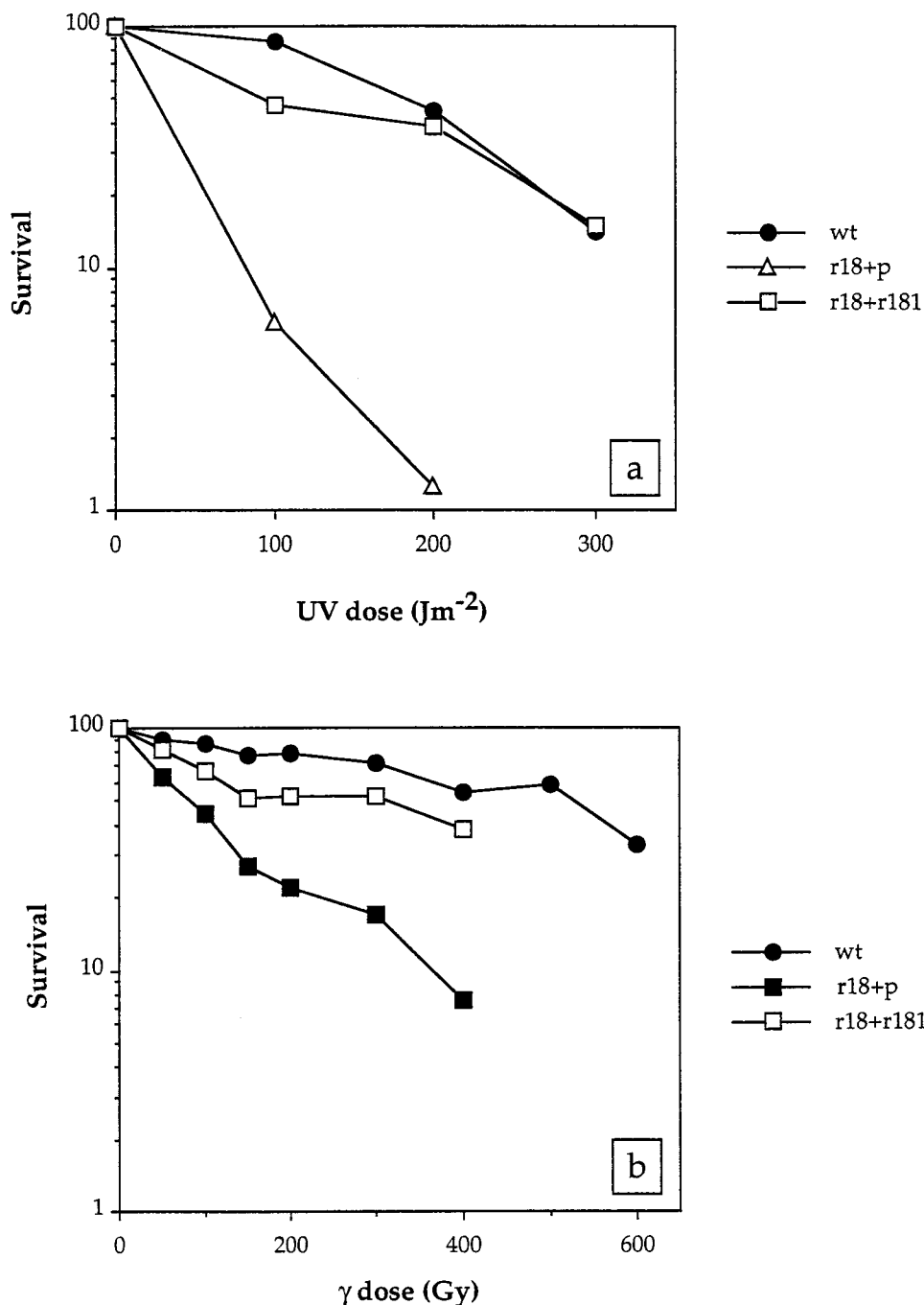
FIG. 5. Survival curves of *rad18* and derivatives. (a) Cells (1,000) were plated and irradiated in a Stratalinker. Note that a Stratalinker reading of 100 J m^{-2} appears to give a lower dose than indicated on the machine. (b) Cells in suspension were exposed to different doses of γ irradiation and then 1,000 cells were plated. wt, wild type; r18, *rad18-X*; r18 + p, *rad18-X* transformed with pUR18N; r18 + r181, *rad18-X* transformed with *rad18* gene in pUR18N (see text).

conserved region close to the C terminus, which may be a Walker type B site also involved in nucleotide binding (43). Alignment of the conserved regions of Rad18 and Rhc18 with previously described members of the SMC family is shown in Fig. 8a and 8b.

The Rad18 product also contains two putative nuclear localization signals (NLSs) at aa 22 to 25 and 41 to 44. The spacing of 16 aa between the basic regions in these potential NLSs is rather longer than the 10 bases which in general

separate bipartite NLSs (37). There is a casein kinase II site (SLEE) about 10 aa upstream of the first basic sequence, as is often found in NLSs (36). A single NLS can be discerned in Rhc18 at aa 35 to 39. The features of the Rad18 and Rhc18 proteins are summarized in Fig. 4b.

Disruption of the *rad18* and *RHC18* genes. At 29°C, proliferating *rad18-X* cells are elongated when compared with wild-type cells (Fig. 9). At 37°C they failed to proliferate. This temperature-sensitive phenotype segregated with *rad18* follow-

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RAD18  MTTELTNVSLEEAITEKTSENRRKRDSDVLQIEEVDLSNVKRIRASRNQDNRPERQSRLQRSSSLIEQVRG   72
RHC18           MISTTISGKRP-----IEQVDDELLSLTAQQENEEQQQQRKRRHQFAPMIQFNSNTLD   54

RAD18  NEDGENDVLNQIRETNSNF---DNRVGVIECIHLVNFMCHDSLKINFGPRINFVIGHNGSGKSAILTGLTIC  140
RHC18  EDSGFRSSSLVATADQDNFLEESPSGYIKKVILRNFMCHEHFELELGSRLNFIVGNNGSGKSAILTAITIG  125

RAD18  LGAKASNTNRAPNMKSLVKQGKNYARISVTISNRGFEAYQPEIYGKSITTERTIRREGSSEYRLRSFNGTV  212
RHC18  LGAKASETNRGSSLKDLIREGCYSAKIILHLDNSKYGAYQQGIFGNEIIVERIIKRDGPASFSLRSENGKE  196

RAD18  ISTKRDELDNICDHMGLQIDNPMNILTQDTARQFLGNSSPKEKYQLFMKGIQLKQLEENY---SLIEQSLI  279
RHC18  ISNKKKDIQTVVDYFSVPVSNPMCFLSQDAARSFLTASTSQDKYSHFMKGTLLQEITENLLYASAIHDSAQ  267

RAD18  NTKNV--LGNKKTGVSYLAKKEEYKLLWEQSRETE-NLHNLLEQKKGEMVWAQVV-------EVEKEILLA  341
RHC18  ENMALHLENLK---SLKAEYEDAKKLLRELNQTSDLNERKMLLQAKS--LWIDVAHNTDACKNLENEISGI  333

RAD18  EKEFQHAEVKLSEAKENIESIVTNQSDIDCKISSKEEVIGRAKGETDTTKSKFEDIVKTFDGYRSEMNDVI  412
RHC18  QQKVLEVTEKIRNRQEKIERYTSDGTTIEAQIDAKVIYVNEKDSEHQNARELLRDVKSRFEKEKSNQAEAQ  404

RAD18  IQKRDIQNSINAAKSCLDVYREQLNTERARENNLGGSQIEKRANESNNLQREIADLSEQIVELESKRNDLH  484
RHC18  SNIDQGRKKVDALNKTIAHLEEELTKE-------------MCGDKDQMRQELEQLEKANEKLREVNNSLV  461

RAD18  SALLENGGNLTSLLTKKDSIANKISDQSEHLKV-LEDVQRDK---VSAFGKNMPQLLKLIT-RETRFQHPI  549
RHC18  VSLQDVKNEERDIQHERESELRTISRSIQNKKVELQNIAKGNDTFLMNFDRNMDRLLRTIEQRKNEFETPA  532

RAD18  KGPMGKYMTVKE--QKWHLIIERILGNVINGFIVRSHHDQLILKELMRQSNCHAIVVVGKY--DPFDYSSG  616
RHC18  IGPLGSLVTIRKGFEKWTRSIQRAISSSLNAFVVSNPKDNRLFRDIMRSCGIRSNIPIVTYCLSQFDYSKG  603

RAD18  EPDSQYPTVLKIIKFDDDEVLHTLINHLGIEKMLLIEDRREAEAYMKRGIANVTQCYALDPRNRGYGFRIV  687
RHC18  RAHGNYPTIVDALEFSKPEIECLFVDLSRIERIVLIEDKNEARNFLQRNPVNVNMALSLRDRRSGFQL---  671

RAD18  STQRSSGISKVTEWNRPPRIGFSSSTSIEAEKKILDDLKKQYNFASNQLNEAKIEQAKFKRDEQLIVE---  755
RHC18  -----SGGYRLDTVTYQDKIRLKVNSSSDNGTQYLKDLIEQ---------ETKELQNIRDRYEEKLSEVRS  728

RAD18  KIEGIKKRILLKRREVNS----LESQELSVLDTEKIQTLRRRISETEKELESYAGQLQDAKNEEHRIRDNQ  822
RHC18  RLKEIDGRLKSTKNEMRKTNFRLTELKMNVGKVVDTGILNSKINERKNQEQAIAS--YEAAKEELGLKIEQ  797

RAD18  RPVIEEIRIYREKIQTETQRLSSLQTELSRLRDEKRNSEVDIERHRQT-----------VESCTNILREKE  882
RHC18  --IAQEAQPIKEQYDSTKLALVEAQDELQQLKEDINSRQSKIQKYKIDTIYYEDKKKKVYLENIKKIEVNVA  866

RAD18  AKKVQCA-QVVADYTAKANTRCERVPVQLSPAEIDNEIERIQMQIAEWRNRTGVSVEQAAEDYLNAIEKHD  954
RHC18  ALKEGIQRQIQNACAFCSKERIENVDLPDIQEEIKRELDKVSRMIQKAEKSLGISQEEVIALIEKCRNKYK  937

RAD18  QAKVLVARITQLLQALEETLRRRNEMWTKFRKLITLRTKELFELYLSQRNFTGKLVIKHQEEFLEPRVYPA  1023
RHC18  EGQKKYMEIDEALNRLHNSLKARDQNYKNAEKGTCFDADMDFRASLKVRKFSGNLSFIKLTKSLEIYLLTI  1008

RAD18  NRNLATAHNRHEKSKVSVQGLSGGEKSFAITCMLLSIWEAMSCPLRCLDEFDVFMDAVNRLVSIKMMVDSA  1094
RHC18  N---------DEKAR-NVDTLSGGEKSFSQMALLLATWKPMRSRIIALDEFDVFMDQVNRKIGTTLIVKKL  1069

RAD18  KDSSDKQFIFITPQDMGQIG--LDKDVVVFRLSDPV-VSSSALPPSTAP                        1140
RHC18  KDIARTQTIIIITPQDIGKIADIDSSGVSIHRMRDPERQNNSNFYN                          1114
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FIG. 6. Alignment of the Rad18 and Rhc18 products. Identities are indicated by black shading; conserved amino acids are indicated by grey shading.

ing back-crossing with rad18+ strains (linkage less than 0.1 centimorgan). At 36.5°C, the cells arrested with a cdc phenotype, namely, elongated with a single nucleus. This cdc phenotype was not evident at 36°C in liquid culture and appeared to be evident on plates only at the very limit of the temperatures at which wild-type cells would proliferate. For this reason, we are unable to attempt a clear characterization of the temperature-sensitive phenotype of rad18. In order to determine if

Rad18 is indeed essential for proliferation, and to study the terminal morphology of rad18Δ null cells, we have deleted both the S. pombe and the S. cerevisiae genes.

To create a deletion construct of the S. pombe rad18 gene, the 6.5-kb KpnI-PstI fragment containing the whole of the rad18 ORF was subcloned from clone F into pUC19. The 3.7-kb AccI fragment (Fig. 3a) was removed and replaced with the ura4 gene as described by Barbet et al. (5). A linear rad18
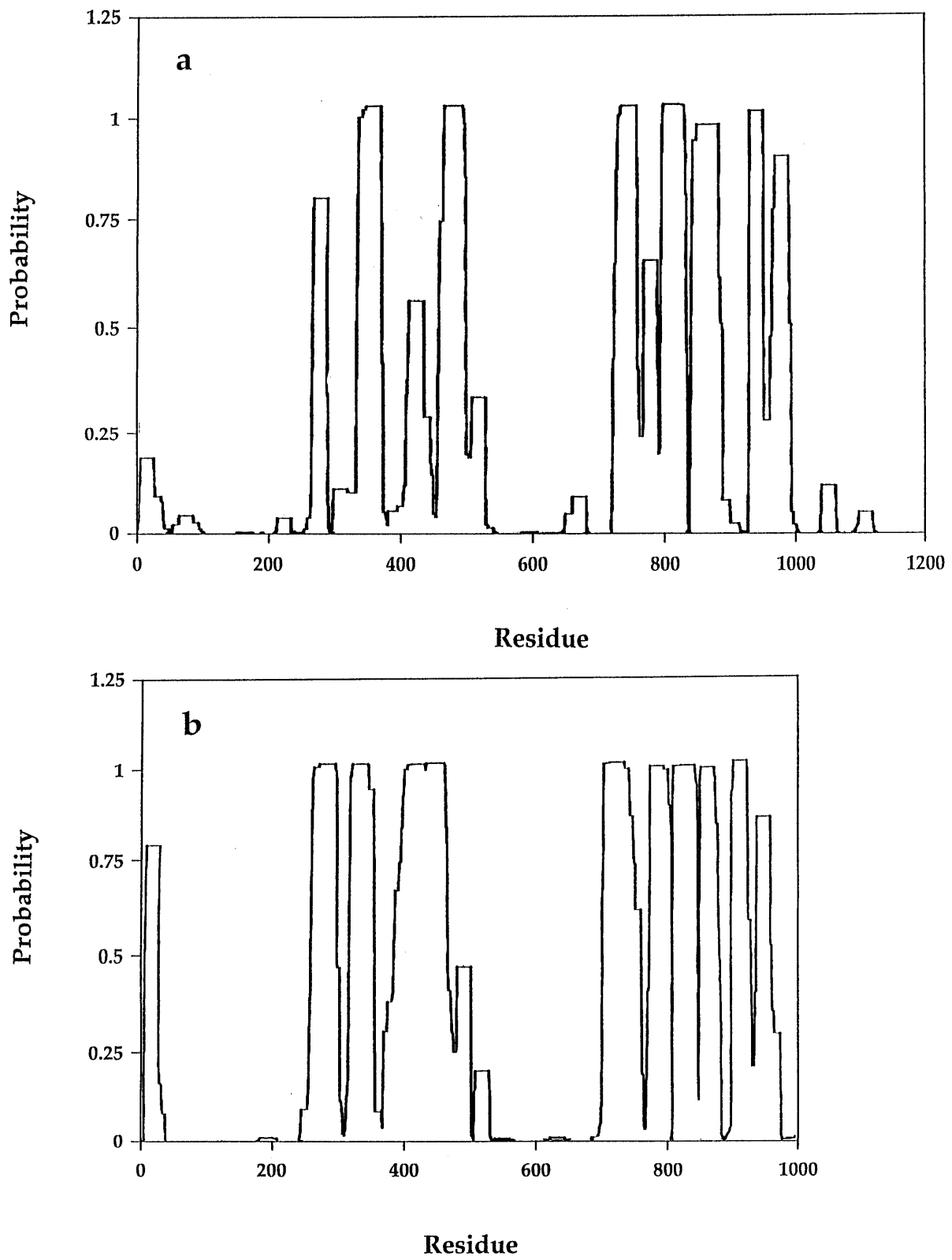
FIG. 7. Coiled coils in Rad18 (a) and Rhc18 (b). The protein sequences were analyzed by using the NewCoil program.
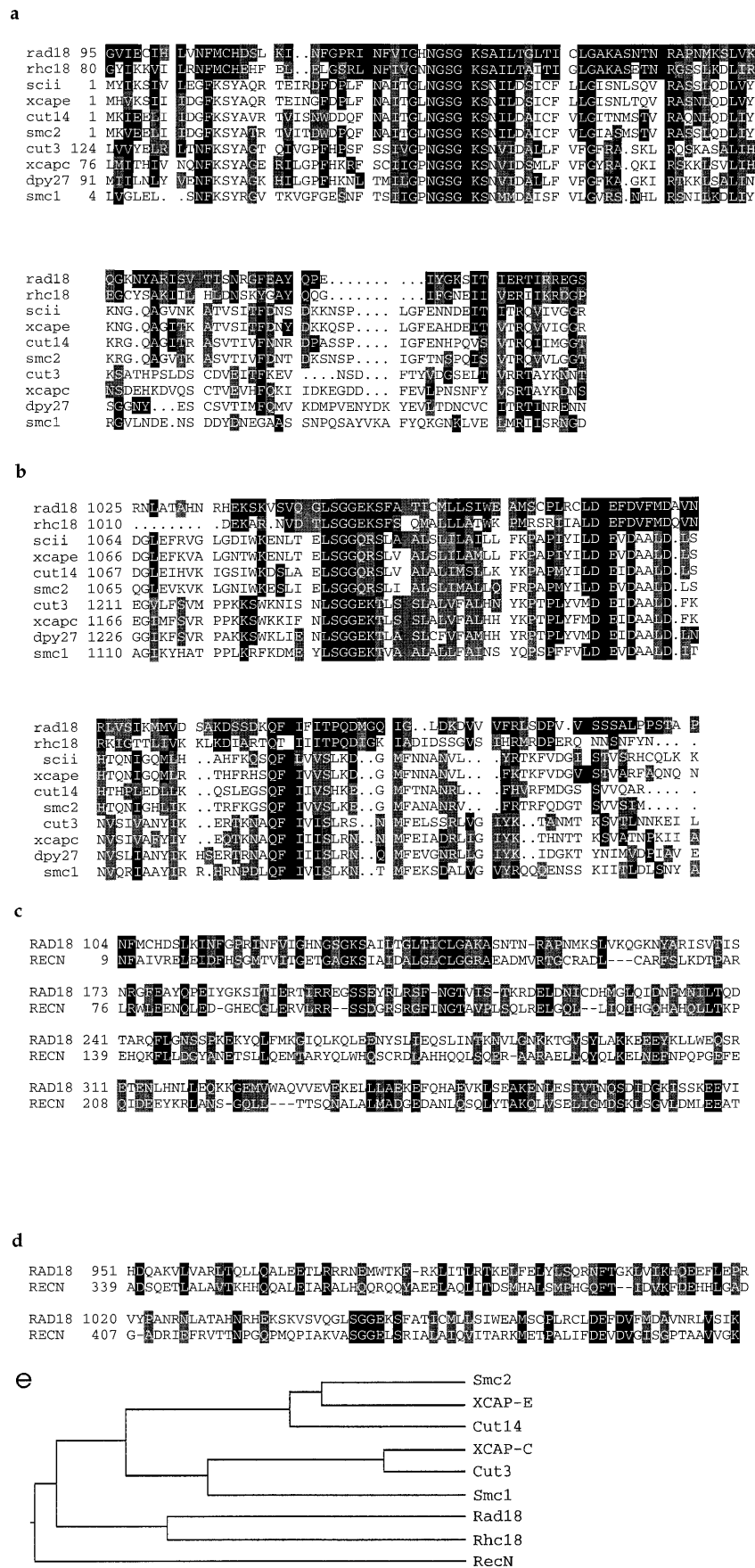
FIG. 8. The SMC family. Alignments of Rad18 and Rhc18 with the N-terminal (a) and the C-terminal (b) conserved sequences of the SMC protein family and the N-terminal (c) and C-terminal (d) conserved domains of *E. coli* RecN. Black shading indicates amino acids identical to Rad18; grey shading indicates amino acids similar to Rad18. (e) Dendrogram of C-terminal conserved regions.
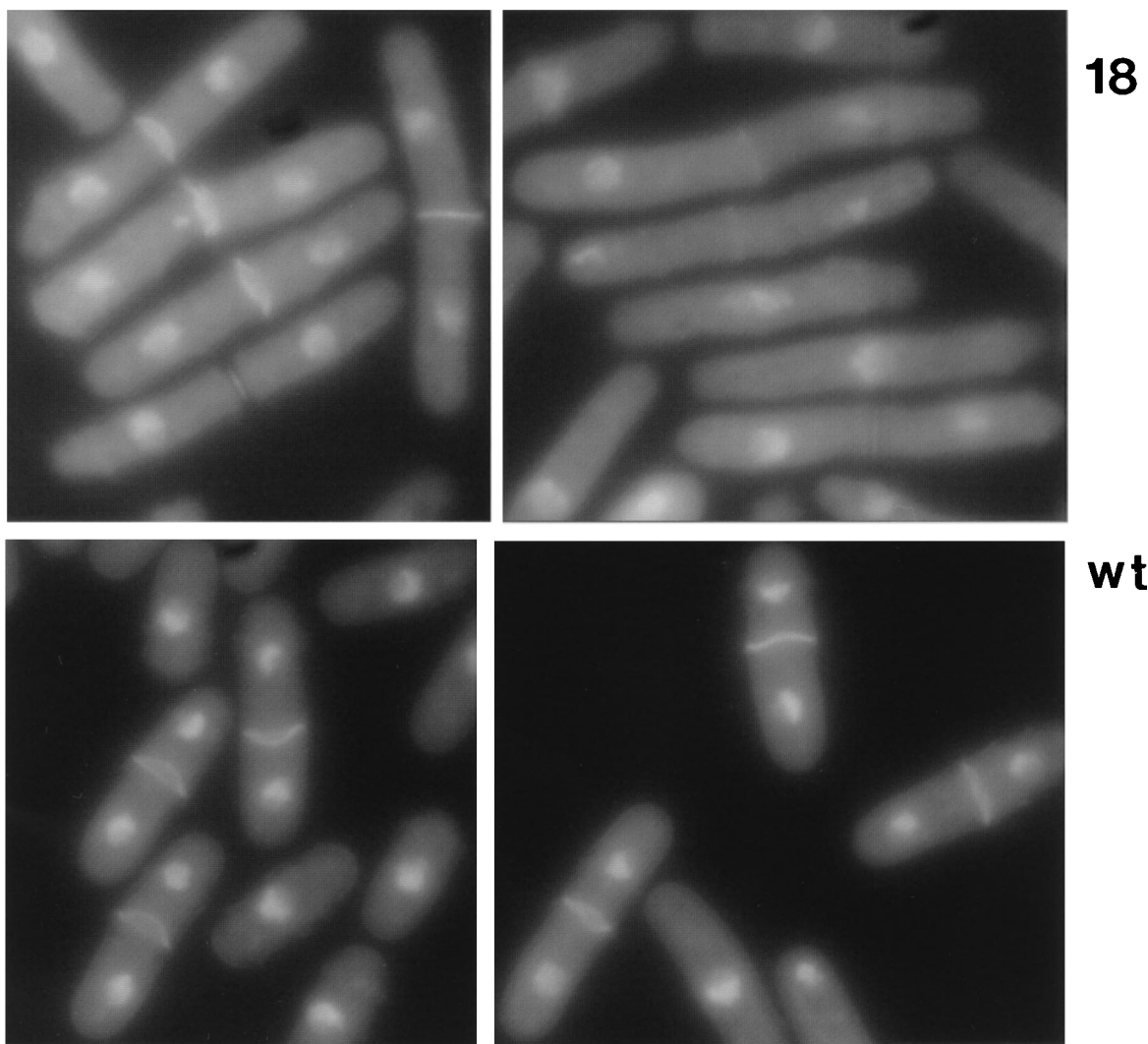
FIG. 9. Exponentially growing cultures of *rad18-X* and wild-type (wt) cells at 29°C.

disruption construct was transformed into *rad18⁺ ura4⁻/ rad18⁺ ura4⁻* diploids. Ura4⁺ transformants were isolated, and in two of them Southern blotting showed that one copy of the endogenous *rad18* gene was replaced by the disrupted *rad18* gene. These two stable ura⁺ transformants were then sporulated and replica plated to ura⁻ plates. No haploid colonies survived in the absence of uracil, suggesting that cells with the disrupted *rad18* gene were nonviable and that the *rad18* gene was essential for cell proliferation. In confirmation, eight tetrads from one of the *rad18⁺/rad18::ura4* diploids were dissected. Only two spores out of each tetrad survived, the surviving colonies all being ura⁻. In order to investigate the terminal morphology of *rad18* null cells, we prepared spores from *rad18⁺/rad18Δ* diploids and germinated them in ura⁻ minimal medium. Spores that germinated progressed through one or two rounds of division before ceasing proliferation mainly as septated cells. DAPI staining did not reveal a "cut" phenotype in these (nonviable) cells.

To create the *S. cerevisiae RHC18* gene deletion construct, a 4.5-kb *Pst*I-*Bam*HI fragment containing the whole of the ORF was cloned into pUC18. A 3.2-kb *Nhe*I-*Hpa*I fragment encompassing most of the coding sequence was replaced with the *S.*

cerevisiae URA3 gene. After transformation into *ura3* diploids, ura⁺ colonies were isolated and checked by Southern blotting. Tetrad analysis of several integrants gave a 2:0 segregation for viability, showing that the *RHC18* gene, like *rad18*, was essential for proliferation. Nonviable spores went through two to four cell divisions, consistent with the results seen in *S. pombe* spore germination experiments.

We have subcloned a fragment containing the entire *S. cerevisiae RHC18* ORF into an *S. pombe* replicating vector containing a leucine-selectable marker. When this plasmid was transformed into a *rad18::ura4⁺/rad18⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-704/ade6-704* diploid strain and the resulting transformants were induced to sporulate, no viable leu⁺ ura⁺ haploids were obtained. Thus, under these conditions, the *RHC18* gene was unable to rescue the essential phenotype of *rad18Δ*.

DISCUSSION

We have cloned and sequenced the *rad18* gene of *S. pombe* and its *S. cerevisiae* homolog *RHC18*. The *rad18* gene has a number of unusual features. (i) It is the only DNA repair gene
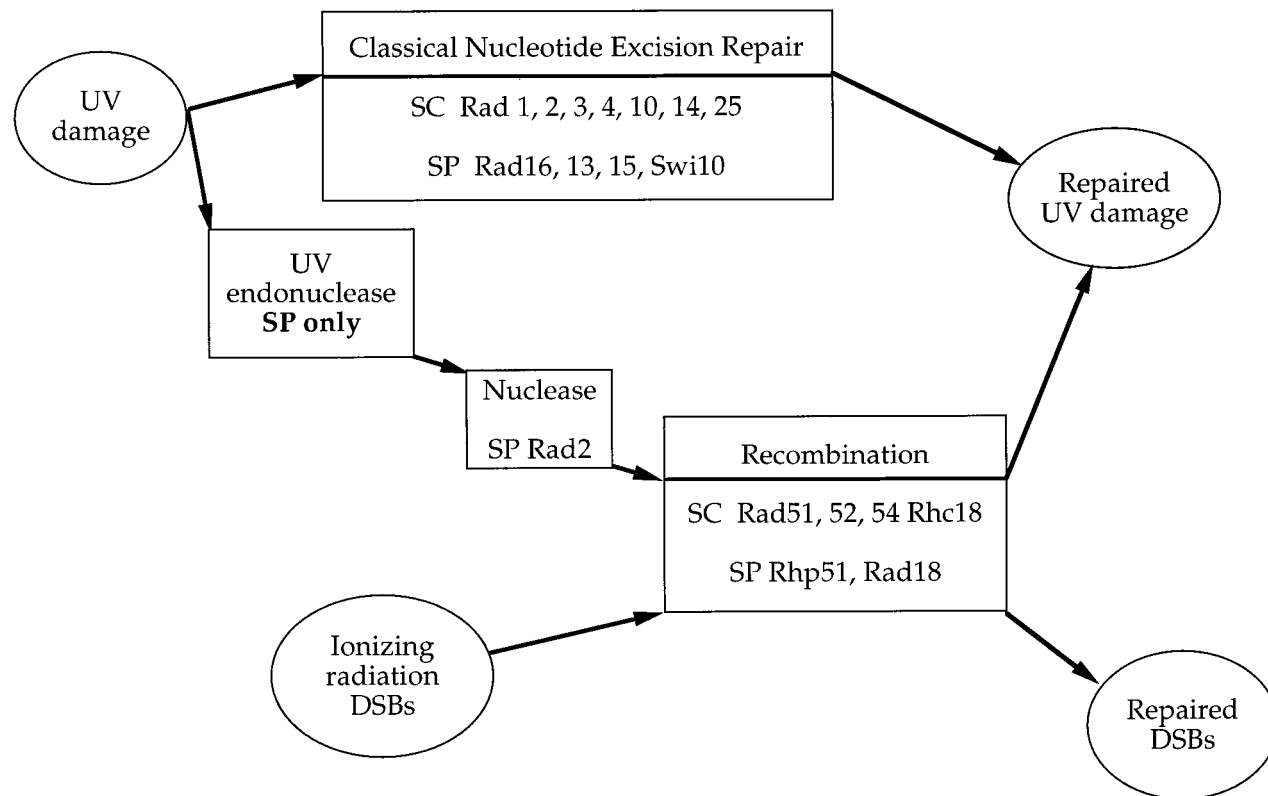
FIG. 10. Model for involvement of Rad18 in excision repair in *S. pombe*. In *S. cerevisiae* UV damage can only be excised by the classical nucleotide excision repair pathway (top). The recombination repair pathway is involved in rejoining of double strand breaks (DSBs) (bottom). We suggest that the presence of a UV endonuclease, only in *S. pombe*, produces a substrate for excision of UV damage by the Rad2 nuclease, followed by a recombination repair pathway (middle).

identified so far which is closely related to the SMC family. (ii) Apart from its role in the response to DNA damage, it has an essential function. (iii) The *rad18* mutant has substantial sensitivity to both UV and γ radiation (Fig. 1). The *S. pombe* NER mutants are sensitive to UV but not γ radiation, whereas the *S. pombe* recombination repair mutants are extremely sensitive to γ radiation and mildly sensitive to UV irradiation. In *S. pombe*, only the checkpoint mutants are highly sensitive to both UV and γ radiation, but the *rad18* mutant shows no defects in cell cycle checkpoints (2, 41).

Removal of damage. Mutants involved in classical NER in *S. pombe* (e.g., *rad13*, *-15*, and *-16* mutants) show only a partial deficiency in the removal of cyclobutane dimers and 6-4 photoproducts, which is consistent with the existence of two overlapping excision pathways in this organism (27). The *rad18-X* mutant also shows a defect in the removal of UV-induced photoproducts (Fig. 2a). The simplest interpretation of the epistasis analysis of *rad18* (Fig. 1) is that Rad18 acts in a pathway for removal of photoproducts that is independent of classical NER and that this second pathway also involves the products of the *rad2* and *rhp51* genes. The double mutants, *rad2 rad18* and *rhp51 rad18*, had UV sensitivities broadly similar to those of the single mutants, whereas double mutants of *rad2*, *rad18*, or *rhp51* with the classical NER deletion mutant *rad13Δ* were much more sensitive than the single mutants.

The known properties of the Rad18, Rad2, and Rhp51 proteins enable us to speculate on the possible mechanism of this second pathway (Fig. 10). Bowman et al. (8) have recently described an endonuclease activity in *S. pombe* extracts that incises DNA immediately 5′ to UV photoproducts. This is a

likely candidate for initiating the process. The mammalian highly conserved homolog of *rad2* (16, 31) encodes a structure-specific endonuclease, called FEN-1, whose preferred substrate is a "flap" structure (15, 16). This protein is also identical to the enzyme DNase IV (38), first isolated by Lindahl (25). Incision of irradiated DNA by the UV endonuclease could provide a substrate for this enzyme to remove a damage-containing DNA fragment. Unlike *rad2*, deletion mutants of the homologous *S. cerevisiae* gene, *RAD27* (or *RTH1*), are hardly if at all sensitive to UV irradiation (35, 49). This is consistent with the idea that this pathway does not operate in *S. cerevisiae*.

The requirement for Rhp51 in this pathway suggests a possible involvement of recombination in the elimination of the UV damage. *rhp51* is the homolog of the *S. cerevisiae RAD51* gene (29, 46). The Rad51 protein forms a nucleoprotein filament with DNA as an early intermediate in recombination (47). We propose, therefore, that the repair intermediate sets in train a recombinational exchange mechanism in which the damage is actually eliminated rather than diluted. We suggest that Rad18 and Rhp51 proteins are involved in a late stage in this process and that in *S. cerevisiae* this pathway does not operate on UV damage, probably because the UV endonuclease does not exist in *S. cerevisiae*.

Postreplicational recombination repair. In *E. coli* double mutants of *recA* and *uvrA* are considerably more UV sensitive than the single mutants. *uvrA* is absolutely required for NER, whereas *recA* is required for postreplication repair, whereby daughter strand gaps opposite UV photoproducts are repaired by a sister strand recombinational exchange process (42). The Rad51 protein of *S. cerevisiae*, and by implication Rhp51 also,

is a homolog of RecA; it forms nucleoprotein filaments very similar in structure to RecA (47) and is likely to function in similar processes. An alternative explanation of our data is that the Rhp51, Rad18, and Rad2 proteins are involved in postreplicative recombination repair. This could be either in addition to their involvement in removal of damage, or alternatively the deficiency in damage removal could be a secondary result of a postreplicative defect. A further possibility is that all three proteins are involved in the second damage removal process, but only Rhp51 is, in addition, involved in postreplication repair. Double mutants of *rhp51* and *rad13* are even more sensitive than double mutants of *rad13* with *rad18* or *rad2*.

Ionizing radiation. Of the three genes discussed above, *rad2* mutants are not sensitive to γ radiation, so the Rad2 (FEN1, DNase IV) nuclease is presumably not involved in repair of damage produced by ionizing radiation. *rhp51Δ*, like the analogous *rad51* null mutant of *S. cerevisiae*, is extremely sensitive to ionizing radiation (reference 29 and Fig. 1d). *rad18* is also sensitive to γ radiation but not as sensitive as *rhp51Δ*. The double mutant has the same sensitivity as *rhp51Δ*. There are two possible explanations. Either (i) Rhp51 and Rad18 are involved in the same repair process but *rad18* is a leaky mutant in this respect (which is quite possible, as we know that it is not a complete loss-of-function mutant by virtue of its viability), or (ii) Rhp51 is involved in two (or more) independent repair processes for ionizing radiation damage and only one of these involves Rad18.

The SMC family. Rad18 and Rhc18 have the same structural features as members of the SMC family. Two coiled-coil regions in the center of the molecule are separated by a spacer region and flanked by conserved globular domains at the amino and carboxy termini (see references 13 and 19 for review). The N terminus contains a Walker A nucleotide binding site (57), and in the C terminus there is a conserved region which has been suggested by Saitoh et al. (43) to form a Walker B site (57), also implicated in ATP hydrolysis. It has been predicted that the molecules form homo- or heterodimers (18, 19, 43). The eight characterized members of the SMC family (Smc1 [51] and Smc2 [50] from *S. cerevisiae*, Cut3 and Cut14 from *S. pombe* [44], XCAP-C and XCAP-E from *Xenopus laevis* [18], SCII from chickens [43], and DPY-27 from *Caenorhabditis elegans* [11]) are all involved with chromosome structure, condensation and/or segregation in mitosis. A detailed analysis of the alignments of all these proteins suggests that Smc2, Cut14, ScII, and XCAP-E are probably true homologs (see reference 50). Apart from shorter C-terminal tails in the two animal genes, the lengths are very similar and sequence conservation is high. Smc1, Cut3, and XCAP-C may also be true homologs, but this is less evident. The structures of the Smc proteins have features in common with myosin and kinesin, leading to the suggestion that they are motor proteins involved in chromosome compaction.

Our finding of an essential function in *rad18* would be consistent with a role in mitotic chromosome condensation, but the properties of *rad18* provide no evidence for a specific function in mitosis. When spores were germinated from a *rad18⁺/rad18::ura4⁺* diploid, the spores containing the deleted *rad18* allele appeared to undergo a normal mitosis as judged by light microscopy. This phenotype is difficult to interpret unambiguously because of the possible persistence of Rad18 protein in the null spores and the fact that it is quite different from the cdc phenotype which appeared to be associated with the *ts* mutant. We were unable to explore the temperature-sensitive phenotype of the *rad18* mutant in detail, as it was observed only in cells grown at limiting temperatures on agar plates and was not observed sufficiently consistently or uniformly within a

TABLE 1. Comparison of the *S. pombe rad18* and *S. cerevisiae RAD50* gene products

Characteristic	Result[a]	
	Rad18	Rad50[b]
Size (aa)	1,140	1,312
Hydrophilic	Y	Y
Nucleotide binding site	Y (aa 122–131)	Y (aa 32–41)
Heptad repeats	Y	Y
Matches to myosin	Y	Y
Sensitivity to γ rays	Y	Y
Sensitivity to UV	Y	Very slight
Essential	Y	N
Recombination deficient	?	Y

[a] Y, yes; N, no; ?, unknown.
[b] Data from reference 1.

single population when cells were grown in liquid medium. We are therefore currently attempting to isolate tighter temperature-sensitive mutants of *rad18* in order to analyze the consequences of loss of *rad18* function in more detail. This is particularly important in view of the fact that the two *S. pombe* SMC family members *cut3* and *cut14* exhibit a "cut" phenotype upon loss of function (i.e., the septum bisects the nucleus, the chromosomes fail to segregate properly, and often DNA can be seen fragmented and strung out along the spindle). However, neither *cut3* nor *cut14* has been implicated in the response to DNA damage (44).

Rad18 and Rhc18, though clearly closely related structurally to the SMC family, are more diverged from the other eight members (see dendrogram in Fig. 8e). We feel therefore that the structure of Rad18 makes it likely that it is a motor protein but that the motor function may be associated with recombination or replication (see below) rather than chromosome condensation and segregation. Such a function has been suggested for the *S. cerevisiae* Rad50 protein, which, although not having all the features of the SMC family, has several properties in common with Rad18 (Table 1). The conserved head and tail structures in Rad18 also show substantial sequence similarity to the *E. coli* RecN protein (40) (Fig. 9c and d), which is required for repair of double-strand breaks (34).

***rad18* and DNA replication.** Our finding that the second repair pathway involves the products of both *rad18* and *rad2* permits us to speculate, on the basis of recent findings with the mammalian homolog of *rad2*, that the essential function of *rad18* may be an involvement with DNA replication. The human homolog of Rad2, as well as being identical to DNase IV, has been shown also to be identical to a nuclease variously designated as MF1 (55) or factor pL (22), which is an essential component for lagging-strand synthesis in the replication of simian virus 40 with purified enzymes (22, 56). These data strongly imply that *S. pombe rad2* also encodes a nuclease involved in DNA replication. Since *rad18* and *rad2* are in the same epistasis group and both show a high level of chromosome loss (31), we propose that the essential function of *rad18* is an as yet unidentified role in DNA replication. (The lack of cell cycle regulation of *rad18* and *rad2* expression [Fig. 3b] might imply that an involvement in DNA replication is unlikely to be their only function.) It is of interest that although *rad2* is not an essential gene, *rad2Δ rhp51Δ* double mutants are inviable (31). This suggests that the *rhp51* group of genes may also have an involvement in replication, perhaps to repair damage-like structures generated during lagging-strand synthesis in DNA replication.

In conclusion, we have shown that the Rad18 protein is

conserved through evolution and that it might be involved in repair of UV damage by a novel pathway involving the Rad2 and Rhp51 proteins and in repair of ionizing radiation damage. Rad18 and Rhc18 represent a subgroup of the SMC superfamily, all other members of which are involved in chromatin architecture and which are proposed to have a motor protein activity. Both *rad18* and its *S. cerevisiae* homolog are essential genes. We propose that *rad18* acts in response to DNA damage possibly coordinating DNA conformation with specific repair events and that its essential role may reflect a related role in DNA replication.

ACKNOWLEDGMENTS

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