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Cloning and Analysis of a Gene Involved in DNA Repair and Recombination, the *rad1* Gene of *Schizosaccharomyces pombe*

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We have cloned the *rad1* gene of *Schizosaccharomyces pombe* by complementation of the *rad1-1* mutant, which is deficient in DNA repair and recombination. The coding region of the gene is 582 base pairs long and contains no introns. The predicted product is a strongly acidic, 22-kilodalton protein containing 194 amino acid residues. This gene does not exhibit significant homology to any other known repair gene. The major transcription start site is at 27 base pairs upstream of the putative start codon. Insertion mutagenesis revealed that besides the coding region, at least 151 base pairs of 5′-flanking sequence are required for full complementing activity. A strain carrying a null allele of *rad1* was constructed and found to have a phenotype closely similar to that of the *rad1-1* mutant. Expression in *Escherichia coli* of the coding region yielded a protein product of a size close to that predicted from the DNA sequence. This product reacted with antibodies raised against a synthetic peptide with a sequence from that predicted for the protein product. We have localized the *rad1* gene to *Not*I fragment E of the *S. pombe* genome.

Schizosaccharomyces pombe is a comparatively radioresistant organism. It is roughly the same cell size and genome complexity as *Saccharomyces cerevisiae*, yet it will tolerate an order-of-magnitude-higher dose of UV or ionizing radiation (34, 47). Thus, *S. pombe* likely has efficient DNA repair systems. It is held that recombinational pathways play a major role in eliminating DNA damage in *S. pombe* (11, 13, 29, 34). Although the predominant life phase in this organism is haploid, *S. pombe*, like mammalian cells but unlike *Saccharomyces cerevisiae*, spends a major part of the cell cycle in G_2, where a sister chromatid is present to provide an intact DNA copy for recombinational repair (34).

Three pathways for repair of radiation-induced DNA damage have been preliminarily identified in *S. pombe* (11, 12, 31, 34). The first, acting both on UV- and gamma radiation-induced damage, requires postirradiation protein synthesis for full efficiency (12). The second, acting on gamma radiation-induced damage only, and the third, acting on UV damage only, require no postdamage protein synthesis (11, 12).

Evidence for the involvement of individual genes in these pathways comes from the collection of radiation-sensitive (*rad*) *S. pombe* mutants, representing 23 distinct loci (23, 31, 41). None of the genes defined by these mutants have been cloned previously. *rad1* mutants are sensitive to both UV and gamma radiation (31) and are not further sensitized by inhibition of protein synthesis (12); hence, *rad1* has tentatively been assigned to the first pathway. Some *rad* mutants, including *rad5* and *rad13*, display an enhanced degree of UV-induced mutability. However, *rad1* cells along with mutants in other loci thought to belong to the same repair pathway have decreased rates of UV-induced mutation (15, 29). These mutants, exemplified by *rad1*, which was the first eucaryotic mutant with this immutable character to be isolated (29, 41), could thus be defective in error-prone repair.

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Caffeine inhibits intergenic recombination in *S. pombe* (24) and also decreases survival after treatment with chemical mutagens or with UV or gamma irradiation (24, 30). *rad1* mutants are refractory to this sensitization by caffeine after exposure to chemical mutagens or UV radiation (but not after gamma irradiation) (30), and so they are thought to be defective in a caffeine-sensitive, recombinational DNA repair pathway. It has been shown that *rad1* mutants have reduced rates of UV-induced recombination between alleles of the same gene on homologous chromosomes (15) and mitotic crossing-over (7), by arguing that the product of *rad1* is involved in recombinational DNA repair.

In view of the predominance of recombinational repair in *S. pombe* and of our interest in understanding the mechanisms of genetic recombination in eucaryotic cells, we have set out to identify genes and gene products involved in this likely multicomponent machinery. The partial complementation of a DNA repair-deficient, UV-sensitive Chinese hamster mutant by the *Saccharomyces cerevisiae* gene *RAD10* (20) and the similarity between a mammalian ubiquitin-conjugating enzyme and the *Saccharomyces cerevisiae RAD6* gene product (17) show that DNA repair mechanisms have to some degree been conserved between yeasts and mammals. The recent finding that the *Saccharomyces cerevisiae RAD2* gene complements the *rad13* mutation in *S. pombe* (26) is a demonstration that the DNA repair systems of these two evolutionarily distant yeasts also have components in common. In addition, the feasibility of directly complementing *S. pombe* mutations with mammalian cDNAs (22) lends further support to our idea that the study of the *S. pombe rad* mutants and genes will provide additional information on the complex enzymology of DNA repair and recombination in higher eucaryotes, including mammals.

MATERIALS AND METHODS

Yeast strains and culture conditions. We used the following *S. pombe* strains: 972h^- (wild type) and h^- *rad1-1 ura4*, provided by A. Nasim h^- *ura4* and h^- *rad1-1 leu1-32 ura4*, which were made by P. Sunnerhagen; h^+ *his3 leu1-32 ura4D18*, a gift from Paul Russell; and Sp629 (diploid h^+

leu1-32 ura4 ade6-210/h⁻ leu1-32 ura4 ade6-216), a gift from David Beach. PS317 (*leu1-32 ura4 ade6*) was obtained by sporulation of Sp629 and was identified on the basis of its adenine auxotrophy. The construction of a strain carrying a *rad1*^{0} allele (PS32r) is described in Results.

Rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) was used for nonselective growth. Minimal medium (MM; 0.67% yeast nitrogen base without amino acids [Difco Laboratories], 2% glucose plus appropriate supplements [75 mg of adenine and 40 mg each of histidine, leucine, and uracil per liter]) was used under selective conditions. For solid medium, 2% agar was added. Cells were grown at 29°C except for matings, which were done on malt extract plates (3% malt extract) at 25°C.

Measurement of survival of yeast strains after UV or gamma irradiation. UV irradiation was with a 15-W Sylvania G15T8 germicidal lamp emitting primarily 254-nm light. The dose rate was 3 W/m^2; radiation intensity was monitored with a Blak Ray J225 short-wave UV meter. Gamma irradiation was performed with cells in suspension, using ^{137}Cs as the radiation source. The dose rate was 1.0 krad/min.

(i) Spot test. For semiquantitative measurements of UV survival, three independent transformant colonies were picked and grown in liquid MM. The cell suspensions were deposited as 5-μl droplets on MM plates and irradiated at various doses ranging from 15 to 270 J/m^2. Survival was recorded after 2 days of growth at 29°C.

(ii) Quantitative measurement of UV or gamma irradiation survival. Cells were grown to an approximate density of 5 × 10^6/ml in YPD or, if transformed with extrachromosomal plasmids, in selective medium (MM). For transformed cells, three independent transformants for each plasmid were pooled. The cells were spun down, resuspended at 10^6 cells per ml with sterile MM, and irradiated. For UV irradiation, the cells were agitated during irradiation. Appropriate dilutions were plated on MM plates (cells transformed with an extrachromosomal plasmid) or YPD plates, and colonies were counted after 3 days of incubation at 29°C.

Preparation of DNA. Genomic *S. pombe* DNA was prepared from cells grown in YPD to stationary phase. After harvest, cells were treated with Zymolyase 20T at 3 mg/ml in 50 mM citrate-phosphate buffer (pH 5.6) for 2 h at 37°C and then lysed with sodium dodecyl sulfate (SDS) at 65°C. Potassium acetate (pH 4.8) was added to 1.25 M, and the sample was kept on ice for 30 min. After centrifugation, nucleic acids were precipitated from the supernatant with 0.6 volumes of isopropanol, and the sample was centrifuged again. The DNA was dissolved in TE (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0], treated with RNase A, extracted with phenol and $CHCl_3$, and ethanol precipitated.

Plasmids were prepared from *S. pombe* essentially as described for genomic DNA except that cells were grown in MM before harvest.

Preparation of RNA. Plasmid-transformed cells were grown to late log phase in liquid MM, harvested by centrifugation, and frozen at −70°C. To the frozen cell pellet was added an equal volume of lysis buffer (50 mM EDTA, 1% SDS), 0.5 volume of glass beads (Sigma Chemical Co.), and 2 volumes of water-saturated phenol. The samples were vortexed at high speed for 3 min and were centrifuged to separate phases. The aqueous phase was extracted twice with phenol-chloroform and once with chloroform and was ethanol precipitated.

Transformation. *S. pombe* transformation was done as described previously (3) except that carrier DNA was omitted.

DNA sequencing. DNA fragments obtained by digestion with restriction enzymes were cloned into the phagemid pTZ18R or pTZ19R (Pharmacia). Alternatively, single-stranded DNA oligonucleotide primers complementary to internal sequences were used to obtain sequence information from some regions. The sequence from both strands was determined from single-stranded templates by the dideoxy-chain termination method of Sanger et al. (40), using 5′-([α-^{35}S]thio)ATP and Sequenase enzyme (United States Biochemical Corp.).

Southern blotting. After gel electrophoresis, DNA was denatured and transferred to nitrocellulose filters (43). Intact plasmids to be used as probes were radiolabeled by nick translation (38), and gel-purified fragments were radiolabeled by random primer extension (9). Hybridizations were carried out at 42°C in the presence of 50% (vol/vol) formamide. Washing was at 65°C in 0.1× SSC (1 × SSC is 150 mM NaCl plus 15 mM sodium citrate)–0.2% SDS for stringent conditions and at 45°C in 0.25 to 4× SSC–0.2% SDS for moderate stringency.

Transcript mapping. Primer extension analysis was carried out with a 17-nucleotide (nt) primer (5′-CCAGCCATTT CCTCTAC-3′) homologous to the 5′ end of the *rad1* gene, or with a 23-nt primer (5′-CATTTAGCTATCCTCATCCTCG G-3′) homologous to the 3′ end. After purification on a 12% polyacrylamide gel, the oligonucleotides were used unlabeled as primers. Approximately 50 μg of total RNA was combined with 20 ng of primer in reverse transcriptase buffer (50 mM Tris hydrochloride [pH 8.3], 25 mM KCl, 20 mM dithiothreitol, 8 mM $MgCl_2$, 250 μM each dATP, dGTP, and dTTP). The mixture was heated to 70°C for 5 min and allowed to anneal at 42°C for 15 min. Unlabeled dCTP was added to 10 μM (with the 5′ primer) or 100 μM (with the 3′ primer) along with 20 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol). The primer was extended with 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) at 42°C for 10 min, cold dCTP was added to 250 μM, and extension was continued for another 20 min.

Construction of a null allele of *rad1*. The 3.4-kilobase-pair (kb) *Bam*HI fragment of p26A (Fig. 1) was inserted into the *Bam*HI site of pTZ18U. The resulting plasmid was linearized with *Bst*XI, and the ends were made blunt with T4 DNA polymerase. The plasmid was then cut with *Bgl*II, and the larger, 5.2-kb fragment lacking the *rad1* protein-coding region was isolated. From pUC19SU4 (a gift from P. Russell), the *S. pombe ura4* gene (2) was isolated on a 1.8-kb fragment in the following way. This plasmid was linearized with *Hin*dIII, and the ends were made blunt with T4 DNA polymerase. A second cut was made with *Bam*HI, and the 1.8-kb *ura4* fragment was isolated and ligated to the 5.2-kb fragment described above, to form pR1u4. The pTZ portion of pR1u4 was released by restriction with *Bam*HI, and the linear DNA was then used to transform Sp629 to Ura$^+$.

RESULTS

Cloning of *rad1* by complementation. A library of genomic *S. pombe* DNA (6) in the high-copy-number replicating vector pFL20 containing the *Saccharomyces cerevisiae URA3* gene (25) was used to transform an *S. pombe rad1-1 ura4* strain to uracil prototrophy. A total of 3 × 10^5 transformants was obtained. Transformed cells were washed off the plates with liquid MM and diluted to a density of 3 × 10^6/ml. Samples of 6 × 10^7 cells were irradiated while agitated in a 140-mm petri dish with 90, 180, or 270 J/m^2 and replated on MM lacking uracil at various densities. Some

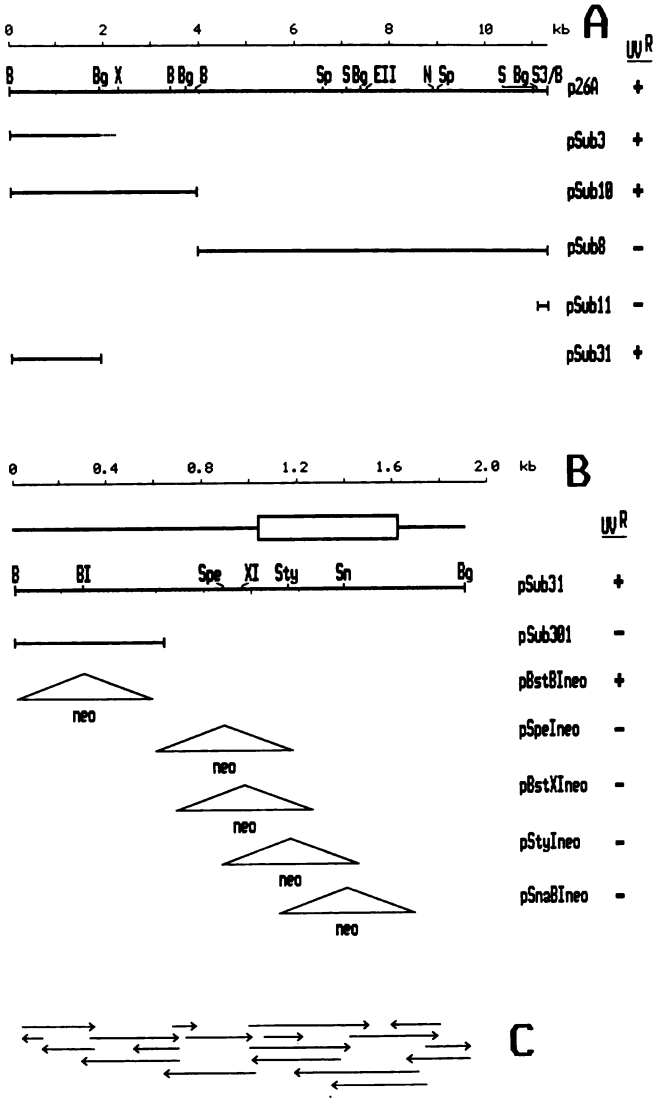

FIG. 1. Physical map of inserts of recombinant plasmids used. Bars indicate sequences still present in the subclones. +, UV resistance of transformants is indistinguishable from that of *rad*$^+$ cells (*h*$^-$ *ura4*) in the spot assay when the plasmid is present in multiple extrachromosomal copies; −, UV resistance of transformants is indistinguishable from that of *h*$^-$ *rad1-1 ura4*. Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; BI, *Bst*BI; EII, *Bst*EII; N, *Nco*I; RV, *Eco*RV; S, *Sal*I; Sn, *Sna*BI; Sp, *Sph*I; Spe, *Spe*I; Sty, *Sty*I; S3, *Sau*3A; X, *Xho*I; XI, *Bst*XI. (A) Structure of the original clone p26A and its derivatives. The dotted line at one end of pSub3 is of unknown origin and is noncontiguous with the rest of insert DNA. (B) Structure of the subclone pSub31 and its derivatives. The ORF is shown as an open box. Direction of transcription is left to right. *neo* insertions in the sequence of pSub31 are shown as triangles directly below the restriction site of insertion, which is also indicated in the name of the corresponding plasmid, given on the right. *neo* insertions not drawn to scale. (C) Sequencing strategy for the pSub31 insert; scale as for panel B.

survivors were expanded in MM, a sample was frozen, and the remainder was spotted onto MM plates and retested for UV resistance. Transformants that consistently showed an enhanced resistance were cultured in nonselective medium for several generations to allow loss of plasmids and were then plated on YPD plates to form distinct colonies. These were replica plated to analyze cosegregation of the *rad* and *ura* markers. Loss of the *ura* marker was always accompanied by loss of UV resistance (76 of 76 cases), indicating that the Rad$^+$ phenotype was caused by a plasmid-borne gene. From UV-resistant isolates, plasmid DNA was prepared and used to transform *Escherichia coli* DH5 to Amp^r. Of 56 plasmids recovered in *E. coli*, six restored UV resistance to the wild-type level on reintroduction into *S. pombe rad1-1 ura4*. Restriction mapping of the six plasmids showed that they were three pairs of sibling clones. The three types of clones had very similar restriction patterns, two of them having 5.2 or 5.5 kb, respectively, of extra DNA at one end of the map. It was concluded that they were all derived from the same chromosomal region. The smallest clone, p26A, with a 11.5-kb insert, was chosen for further study.

Plasmid p26A contains the *rad1* gene. The 2.2-kb *Sal*I-*Xho*I fragment of Yep13 (4) containing the *Saccharomyces cerevisiae LEU2* gene, which complements the *leu1-32* mutation in *S. pombe*, was inserted into the *Sal*I site of pUC19, creating the nonreplicating vector pLEU2. The *Saccharomyces cerevisiae LEU2* and *S. pombe leu1* genes are sufficiently divergent to prevent either cross-hybridization under stringent conditions or plasmid integration by homologous recombination at the *leu1* locus. The 3.4-kb *Bam*HI fragment from p26A, containing an internal *Xho*I site (Fig. 1), was then cloned into the *Bam*HI site of pLEU2, to form pBLEU2. At its unique *Xho*I site, pBLEU2 was linearized and used to transform *S. pombe h*$^+$ *his3 leu1-32 ura4D18* to Leu$^+$. From transformants that showed a stable leucine prototrophy, genomic DNA was isolated, restricted with *Nco*I, electrophoresed, and blotted onto nitrocellulose. Labeled pBLEU2 was hybridized to this blot. In the integrants, a single band hybridized, migrating slower than the single band seen in DNA from the parental *h*$^+$ *his leu1-32 ura4D18* strain (not shown). Since *Nco*I does not cut pBLEU2, this result indicates that integration into the *S. pombe* genome had indeed taken place by homologous recombination and that pBLEU2 had integrated only at the chromosomal locus from which p26A was derived. Two independent integrants were crossed to a *h*$^-$ *leu1-32 rad1-1 ura4* strain, and segregation of the *rad*, *his*, and *rad1 int*::*LEU2* (*LEU2* integrated at the *rad1* locus) markers was monitored by random spore analysis. In both crosses, tight linkage was found between the *rad* and the *rad1 int*::*LEU2* markers. In one of the crosses, 186 nonsegregants (Rad$^+$ Leu$^+$, Rad$^-$ Leu$^-$) and 1 segregant (Rad$^-$ Leu$^+$) were found; in the other, 184 nonsegregants and 2 segregants (Rad$^+$ Leu$^-$, Rad$^-$ Leu$^+$) were found. Given the approximate relationship 1 centimorgan = 6 kb in *S. pombe* (8), this overall recombination frequency of 0.8% is within the range expected from the distance between the *rad1* gene and the *LEU2* gene in the integrants. No linkage was found between *his3* and any of the other loci.

Localization of *rad1* within p26A. Subclones of p26A were constructed by complete or partial digestion of the 11.5-kb insert with *Bam*HI, *Bgl*II, or *Sau*3A and insertion of these fragments into the *Bam*HI site of pFL20. It was noted that only a small subset of the intended subclones was obtained, indicating the possibility that some configurations of sequences in the *rad1* locus cause instability in *E. coli* when cloned in certain vectors. Similar problems have been reported for other *S. pombe* loci (5). Of the subclones obtained, two (pSub3 and pSub10) retained full complementing activity when reintroduced into *h*$^-$ *rad1-1 ura4* cells (Fig. 1A and 2). Since these two subclones were derived from the same region of p26A (Fig. 1A), the smallest, pSub3, was further characterized. In the course of experiments, it was

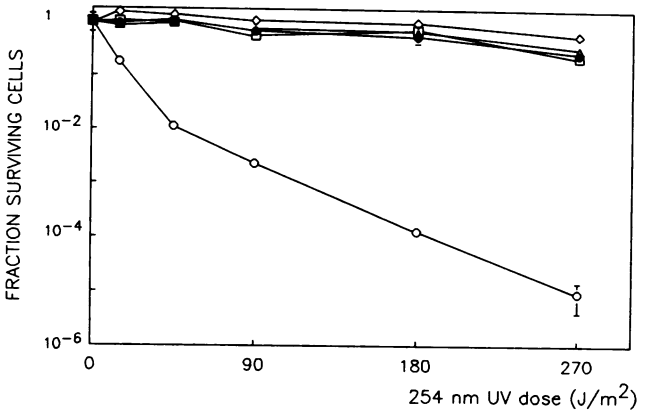

FIG. 2. Survival after UV irradiation of strains mutant in *radl* (*h⁻ radl-1 ura4*) and wild-type (*h⁻ ura4*) transformed with plasmids containing or lacking a *radl* insert. Cells were grown and irradiated as described in Materials and Methods. Each datum point is the average of at least two plates. Error bars represent ±1 standard deviation from the mean. Symbols: ○, *radl* cells transformed with pFL20; ●, wild-type cells transformed with pFL20; □, *radl* cells transformed with p26A; △, *radl* cells transformed with pSub3; ◇, *radl* cells transformed with pSub31.

found that pSub3 contains 0.3 kb of sequence beyond the *Bgl*II site that is not colinear with p26A, from which it was derived (Fig. 1A). Therefore, the 1.9-kb *Bam*HI-*Bgl*II fragment from the left-hand end of the insert of p26A was cloned into the *Bam*HI site of pFL20, forming pSub31, and was grown in *E. coli* DH5, in which it was found to be stable. To verify that full complementing activity had been preserved during these subcloning steps, the survival after UV irradiation of various transformants was quantitatively measured. pSub31 as well as pSub3 and the original clone p26A all conferred UV resistance to *radl-1* cells to the same level as that of wild-type cells (Fig. 2).

Because of the presence of an unidentified DNA fragment in pSub3, we wanted to ascertain that no other rearrangements had taken place in the course of subcloning. Detailed restriction mapping and Southern hybridization analyses demonstrated that p26A was colinear with the *S. pombe* genome. Likewise, pSub31 was found to be colinear with the corresponding parts of p26A and pSub3 (Fig. 3 and data not shown).

The Southern analysis data also revealed that the *radl* gene is present as a single copy in the *S. pombe* genome and that no gross alterations could be detected in the *radl* locus of *radl-1* cells compared with that of the wild type (Fig. 3 and data not shown).

Sequence of the *radl* gene. The nucleotide sequence of the entire 1,905-base-pair (bp) insert of pSub31 was determined (Fig. 4). Analysis of all six possible reading frames reveals one open reading frame (ORF) of 582 bp from bp 1026 to 1607. Computer programs designed to identify protein-coding stretches of DNA (45) indicate a high likelihood of protein-coding throughout this 582-bp ORF. The first possible start codon in this ORF has the sequence context GTAG AGGAAATGG, which fits the consensus rule among eucaryotic start codons that there should be a G at positions −9, −6, and +4 and a purine at −3 (18). The putative protein encoded by this ORF, assuming that the first ATG is used, consists of 194 amino acids and has a molecular weight of 21,813.

Many *Saccharomyces cerevisiae RAD* genes have strongly hydrophilic amino and carboxy termini (10). In the

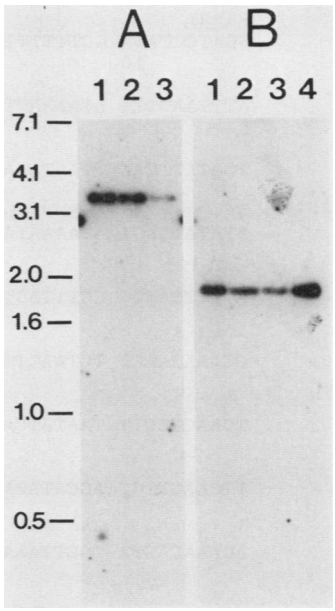

FIG. 3. Southern blot analysis of the genomic *radl* locus and of clones derived from it. Genomic or plasmid DNA was restricted, run on a 0.7% agarose gel, blotted, and hybridized, using the 1.9-kb *Bam*HI-*Bgl*II insert fragment contained in pSub31 as a probe. Sizes of molecular weight markers are given in kilobases on the left. (A) DNA digested with *Bam*HI. Lanes: 1, 972*h⁻*; 2, *h⁻ radl-1 ura4*; 3, p26A. (B) DNA digested with *Bam*HI and *Bgl*II. Lanes: 1, 972*h⁻*; 2, *h⁻ radl-1 ura4*; 3, p26A; 4, pSub3.

radl protein, the most polar region is found toward the carboxy terminus. A prominent feature of the amino acid sequence of the *radl* protein is the acidic domain near the carboxy terminus. From amino acid residues 156 to 165 there are 10 consecutive acidic amino acid residues, and 6 of the 9 last residues are acidic (Fig. 4). The calculated net charge of the protein at pH 7 is −28, an unusually high negative charge for a protein of this small size. There are 14 basic amino acid residues in the *radl* protein. Of these, six are found in a stretch from residues 96 to 122 that contains no acidic amino acids. Thus, this middle portion of the strongly acidic protein contains a positively charged region.

At position 941, the sequence TATAAT is found. Because of the high A+T content of *S. pombe* DNA, one can find several sequences reminiscent of TATA motifs in the 5′-flanking region; however, TATAAT conforms exactly with the derived consensus for yeast promoter TATA boxes (10) rather than the TATAAAT seen in higher eucaryotes.

Functional localization of *radl* within pSub31. To delineate the *radl*-complementing activity in the 1.9-kb insert of pSub31, subcloning was attempted by inserting either partial or complete *Sau*3A digestion products in the *Bam*HI site of pFL20. Again, apparent stability problems prevented recovery of most subclones. Only one *Sau*3A fragment was obtained (Fig. 1B). As an alternative approach, the sequence was interrupted by insertions at various locations. The 1.4-kb *Sma*I-*Hin*dIII fragment from pSV2neo (44) containing the Tn*5* neomycin resistance (*neo*) gene was excised, and the ends were filled in with T4 DNA polymerase. Plasmid pTZSub31, in which the *Sma*I-*Sal*I fragment of pSub31 consisting of the *S. pombe* DNA plus 0.6 kb of flanking pFL20 sequence had been inserted between the *Sma*I and *Sal*I sites of pTZ19R, was linearized at various unique sites (Fig. 1B), and the ends were made blunt with T4 DNA

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BamHI
GGATCCTATA GCTTTTTTGA ATATCATTTG GTAAATTCAA ATATGGCTCT TAAAACTTGT
        10          20          30          40          50          60

ATTTAATTTG ATAAAATTTC TGTGTCGAGA GATAAATAAA TAAAGACAGT TAGAAGTAGA
                                                                   120

TGCTATTCAA TTAATAAATA AAATACTCGT CTATTAAGCT TTACGGCTTT CCAAAAATTT
                                                                   180

ATATATCAGA ATAAAATATT TCACAATTTT TTTTTTTTTA AAAAAGAAAA AGCAATCTCA
                                                                   240
                                                        BstBI
ATTTTCATTT CCTTTAGTAA TCTGCATACC TTTTAAAATT GTGCACCTTT TCGAATTTTA
                                                                   300

CTAAATAACT TCTAACTTGA ACTGTCTTTC TTATTCTGCG TTTGGTCTTG TAATCAAGTG
                                                                   360

TCAAGTCCTT TAATATCAGA AGGAAAAATT ATCAATTTAT AAGATTAGCA TTTACGGGTG
                                                                   420

TTCAAGTTGT AGCATATAAC CAATTTTTTA TTGTTGGACG TAAATTCGCA TGTTTCAAGC
                                                                   480

AGAAACAGTA TGCTTAAAGC AGATTCAGTC AACATTACGA TGCATTGATT TTTCAAAGGA
                                                                   540

ATGTACTATC GAAATCACAA GTCGTGGACT ACGGTTTGCA GTGGAGGAAT CGCAGTCTTT
                                                                   600

GCAGGGTAAG ACAAGTCGAG TCTCACATCT ATGATCGTTA AAAAATTTGA AGCTACTTAA
                                                                   660

TAAAGTTTCA TATATTAATG CGATTCTTGA AATGCATTCA CTTTTTCTTT ATTGTTCACT
                                                                   720

CACACATTAA GCTCACGCCT TTCTTGATAA GTCGTTGTTT CAAACGTTTA ATTTTCAGGG
                                                                   780

TGATTCAGAT GGGGATACAT ATATGTTCCA GACGATGATT TCACCTCTTT TACAATCATT
                                                                   840
                                                  SpeI
ATCAATTTAT ACAGATGGAA AAGAACGGAT TTCAACTAGT GCCTGGGATC AACCCACTGT
                                                                   900
                                                                  --
AAACATAATG CACAAACGAG GCGTTATCTG CAAAGTACAG TATAATGGAC CCGGCTGTCC
                                                                   960
   BstXI                                          |------>
ATTTATATGG GAGTAAGGAT TTTATCATTA ACATTCTGTA CTGACAAAAA TAAAGAGTAG
                                                                  1020
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FIG. 4. Nucleotide sequence of the *rad1* gene and predicted amino acid sequence of the protein product. Relevant restriction sites are shown directly above the corresponding sequence. Start and stop codons of the ORF are shown in boldface and underlined. The putative TATA box is underlined. The major transcription initiation site is indicated with an arrow above it. The arrow indicates the direction of transcription, the base of the arrow being located above the first nucleotide of the transcript. The amino acid sequence is shown in boldface, with each amino acid residue directly above the corresponding nucleotide triplet.

polymerase. The resulting linear plasmids were ligated to the 1.4-kb *neo* fragment, and used to transform *E. coli* to ampicillin and neomycin resistance. Finally, the *rad1* region with the respective *neo* insertions was moved back into pFL20 by insertion as a *Sma*I-*Bam*HI fragment between the *Sma*I and *Bam*HI sites of pFL20. The constructs carrying the *neo* insertions were then transformed into the *rad1-1* mutant, and complementation of the Rad phenotype by these extrachromosomal plasmids was scored. Insertions in the ORF (pStyIneo and pSnaBIneo) left no detectable residual activity (Fig. 1B). Also, in insertions in the 5′ noncoding region at either 60 bp (pBstXIneo) or 151 bp (pSpeIneo) upstream of the putative start codon, activity was completely abolished. In contrast, insertion further upstream, 734 bp from the start codon (pBstBIneo), had no apparent effect on UV resistance.

Transcripts from *rad1*. Primer extension mapping using a primer complementary to the 5′ end of the ORF pointed to nt 999 as the only major start site of the *rad1* transcript (Fig. 5). This site is 53 nt downstream of the TATA motif at bp 941 to 946, in the same range as previously determined distances from TATA boxes to transcription initiation sites in *S. pombe* genes (39). The initiating nucleotide of the transcript would then be a U for the major start site. There are no start codons in the leader sequence upstream of the presumed functioning initiation codon at 1026.

Using a primer complementary to the 3′ end of the ORF, a reverse transcription product was obtained that was of a size consistent with a transcript originating at nt 999 and being colinear with *rad1* DNA at least to the 3′ end of the ORF, at nt 1610 (data not shown). This product was not seen in reactions done in the absence of primer.

Phenotype of the null allele of the *rad1* gene. After transformation of the diploid strain Sp629 with *Bam*HI-digested pR1u4 (Fig. 6A), stable transformants were analyzed by Southern blotting, and clones were identified that contained

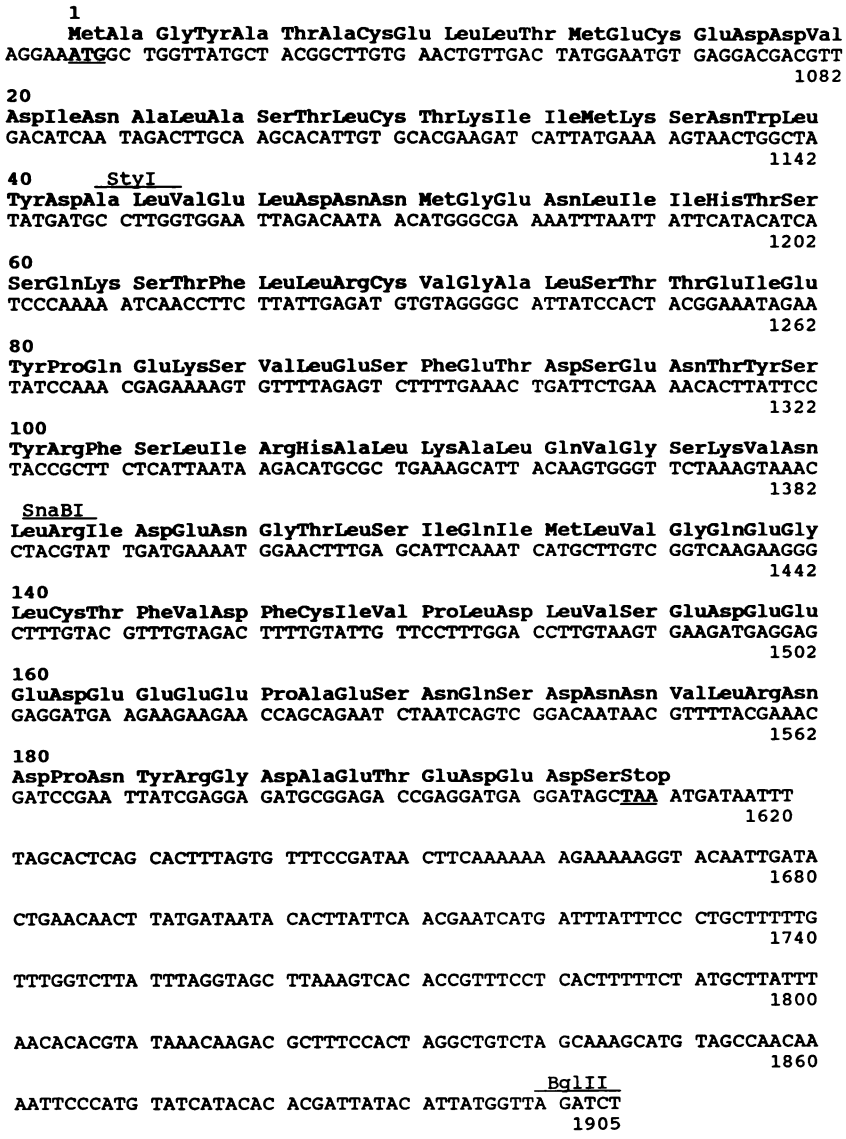

FIG. 4—*Continued.*

the 4.3-kb *Eco*RV band predicted for the *rad1* null allele (Fig. 6B). Additional Southern analysis with other restriction enzymes confirmed the interpretation that the *ura4* gene had been inserted at the *rad1* locus; also, using the *ura4* fragment as a probe, it was demonstrated that this is the only wild-type copy of the *ura4* gene in these transformants (data not shown). Haploid derivatives containing the $rad1^0$ allele from one diploid transformant were obtained by sporulation, and one strain (PS32r) with the genotype *leu1-32 ade6 rad1::ura4* was studied further.

In some cases, the phenotype associated with a null mutation is much more pronounced than that of a point mutation, indicating leakiness of the latter. In the *RAD10* gene of *Saccharomyces cerevisiae*, for example, this was manifested as an increase in sensitivity toward UV light of several orders of magnitude in the strain carrying the null mutation over the level of the strain carrying the point mutation (47).

For the *rad1* gene, however, there was little if any phenotypic difference between the original *rad1-1* allele and the *rad1::ura4* null allele. To rule out any nonspecific strain-to-strain variability when assessing the relative effects of the two *rad1* alleles, the survival rate of the strain carrying the $rad1^0$ allele (PS32r) was compared with that of an isogenic rad^+ strain (PS317; see Materials and Methods). The survival rate of this strain upon irradiation was indistinguishable from that carrying the other *rad1* allele. This is true also if the cells were plated on medium containing caffeine after irradiation (Fig. 7B and D).

The enhanced lethality previously described for rad^+ cells (24, 30) on caffeine-containing medium after exposure to UV (Fig. 7A versus B) or gamma (Fig. 7C versus D) radiation was seen for both rad^+ strains, as expected. For *rad1-1* cells, there was no enhancement of lethality by caffeine after UV irradiation (24); this observation was confirmed here and was also borne out for the $rad1^0$ allele (Fig. 7A versus B). After gamma irradiation, however, there was a residual caffeine effect also in *rad1-1* cells (30); again we observed the expected pattern for *rad1-1* cells and identical behavior for cells containing the $rad1^0$ allele (Fig. 7C versus D).

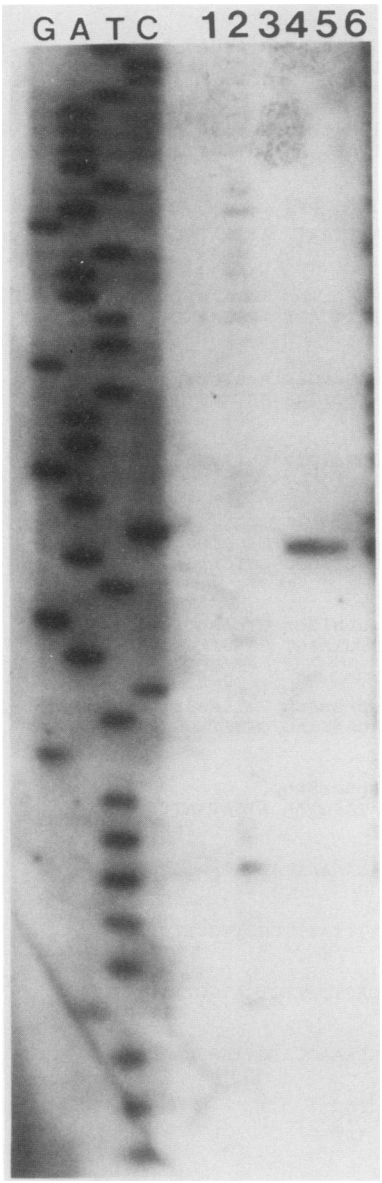

FIG. 5. Primer extension analysis of the 5′ end of the *rad1* transcript, using a 17-bp primer complementary to the 5′ end of the ORF. Lanes: G, A, T, and C, dideoxy sequencing lanes; 1, extension products from *Saccharomyces cerevisiae* tRNA (Sigma Chemical Co.), no primer added; 2, as in lane 1 but in the presence of primer; 3 to 6, primer extension products obtained by using RNA from *S. pombe h⁻ ura4* cells transformed with plasmids (p26A, no primer added [lane 3], as in lane 3 but in the presence of primer [lane 4], pFL20 [lane 5], and as in lane 5 but no primer added [lane 6]).

Identification of the *rad1* protein product by expression in *E. coli*. A peptide with the sequence Cys-Glu-Ile-Glu-Tyr-Pro-Gln-Glu-Lys-Ser was synthesized and coupled to keyhole limpet hemocyanin through its free thiol group, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. The conjugate was used to immunize rabbits.

At positions 1023 to 1028, the nucleotide sequence of the *rad1* gene in pSub3 was changed to introduce an *Nde*I site (CATATG) at the start codon, using a 42-nt synthetic oligonucleotide as described previously (19). From this altered plasmid, a 0.9-kb *Nde*I-*Bgl*II fragment containing the protein-coding region was excised and ligated between the *Nde*I and *Bam*HI sites of pT7.7 (46), putting the *rad1* coding region under control of a T7 promoter. The resulting plasmid was used to transform *E. coli* BL21 harboring plasmid pLysS (a gift from F. W. Studier) to Amp^r. After induction of T7 RNA polymerase with isothiogalactopyranoside (IPTG), proteins were analyzed by gel electrophoresis and immunoblotting.

By comparing the patterns of immunoreactive bands obtained with preimmune and immune sera (Fig. 8), it is possible to identify unequivocally the protein band with an apparent molecular size of 23 kilodaltons (marked with an arrow in Fig. 8) as the only species reacting with immune serum only. Furthermore, this species was not seen before induction with IPTG (Fig. 8B, lane 1).

Subchromosomal localization of *rad1*. *rad1* has been assigned to the short arm of chromosome I, distal to *aro3* (16). Recently, a *Not*I restriction map of the entire *S. pombe* genome was established (8). Since *aro3* maps to *Not*I fragment E, the penultimate fragment on the short arm of chromosome I (8), *rad1* would be predicted to be either in fragment E or in the telomeric fragment, I. When pSub31 was used to probe *Not*I-digested genomic DNA, hybridization to a 1 Mb band was seen (not shown), placing *rad1* in *Not*I fragment E.

DISCUSSION

The conclusion that the coding region of the *rad1* gene is contained between bp 1026 and 1610 of the sequence presented here and is translated to a 22-kilodalton product is based on the following evidence.

Besides the 582-bp ORF, there are no ORFs longer than 172 bp in the sequence in any of the six reading frames. By insertional mutagenesis in the coding region, this 582-bp ORF has been demonstrated to be essential for restoration of UV resistance. Within this ORF, there is no combination of donor, branch, and acceptor sites (27) that would allow a splice of the transcript to occur without altering the reading frame and truncating the predicted protein product. In eucaryotic genes, the first available ATG is almost always used for initiation of translation (18). The first in-frame ATG in the 582-bp ORF, at 1026, has a G at positions −9, −6, −3, and +4 and would thus make a good start codon (18). The second, at 1062, has a purine at −3 and a G at +4, whereas the guanines at −9 and −6 are lacking. Hence, it would appear less strong as a start codon than the first, making it likely that the start codon at 1026 is predominant, yielding a protein product of 22 kilodaltons.

The size of the observed protein product in an *E. coli* expression system also fits this prediction. For the *RAD6* protein of *Saccharomyces cerevisiae*, the mobility in denaturing gel electrophoresis is considerably lower than expected from the calculated molecular weight (28). This finding has been ascribed to the long polyacidic tail at the carboxy terminus, which may protrude from the globular moiety of the protein. If such an effect exists for the *rad1* protein, it must be much smaller, since the deviation between expected and observed mobility is only slight. Likewise, the number of consecutive acidic residues in the carobxy-terminal part of the *rad1* protein is lower than in the *RAD6* protein.

Formally, it could be argued that in addition to the 582-bp ORF, another gene, e.g., of regulatory function, might be required for *rad1* function. Since insertions at *Spe*I or *Bst*XI, upstream of the 582-bp ORF, destroy function, this could be taken as evidence that the 172-bp ORF from bp 804 to 976 is

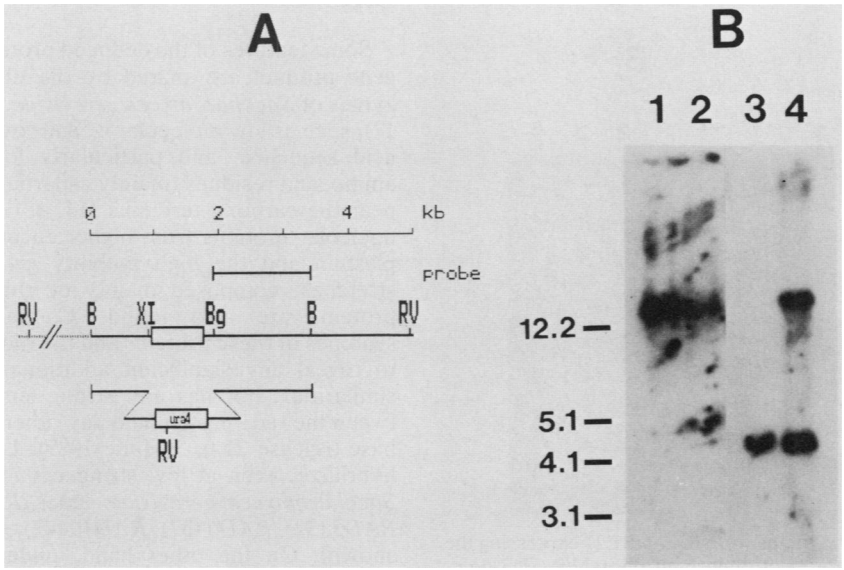

FIG. 6. Construction of a null allele of *rad1*. (A) The chromosomal *rad1* locus; symbols and abbreviations are as for Fig. 1. The 1.5-kb *Bam*HI-*Bgl*II fragment used as a probe in the Southern analysis in panel B is shown above the corresponding region of the locus. The 4.3-kb *Bam*HI fragment from pR1u4 containing the *ura4* gene used to transform Sp629 is shown directly below the homologous region of the chromosomal locus; the offset portion indicates the region of nonhomology. Dotted line to the left indicates uncloned DNA on the 5′ flank of the *rad1* locus. (B) Southern analysis of a strain containing the $rad1^0$ allele. Genomic DNA was restricted with *Eco*RV (RV). Lanes: 1, 972h^-; 2, Sp629; 3, haploid strain (PS32r) containing the $rad1^0$ allele; 4, diploid strain (original transformant) containing the $rad1^0$ and wild-type *rad1* alleles. Sizes of molecular weight markers are given in kilobases on the left.

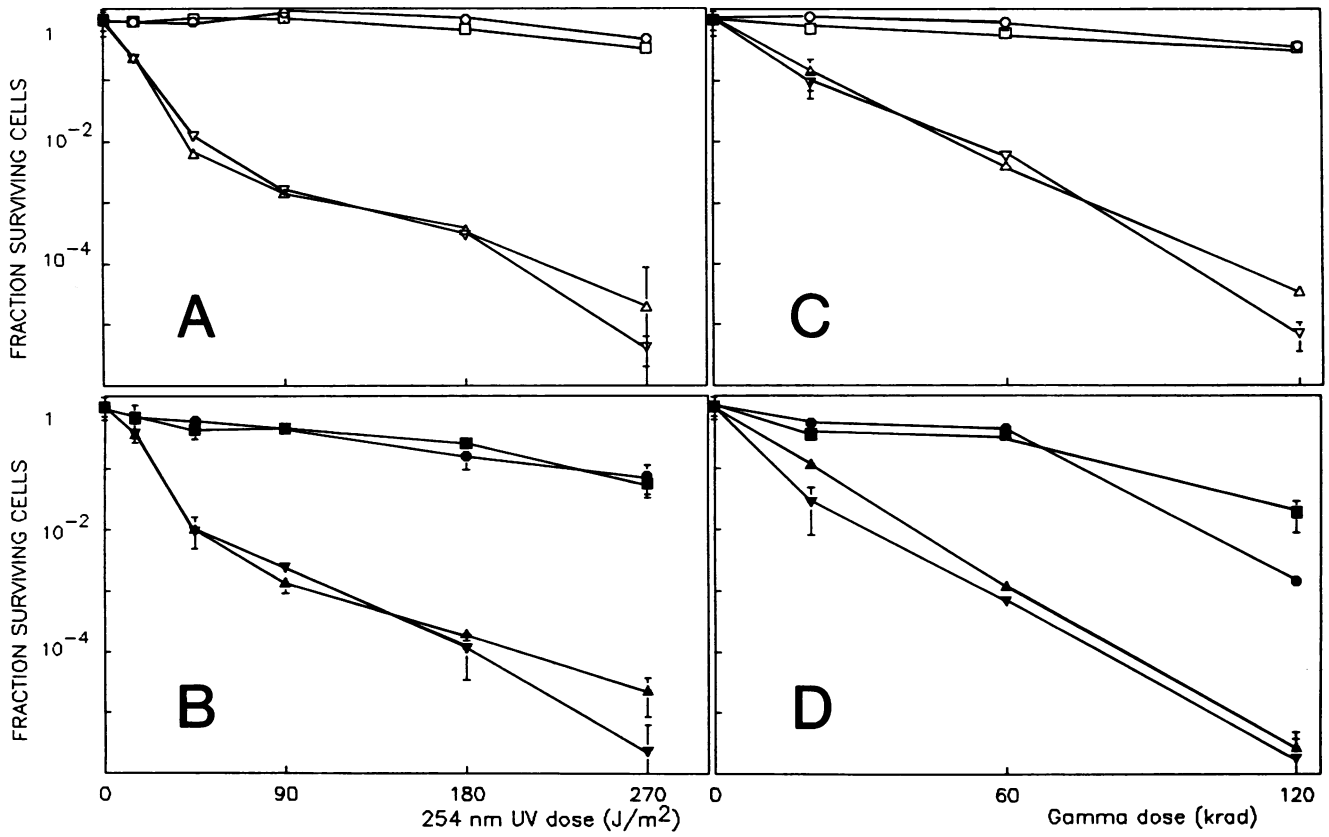

FIG. 7. Survival after UV or gamma irradiation of wild-type cells and of cells containing the *rad1-1* or $rad1^0$ allele. Cells were grown and irradiated as described in Materials and Methods and were plated on YPD (open symbols) or YPD plus caffeine (1 mg/ml; filled symbols) after irradiation. Each datum point is the average of at least two plates. Error bars represent ±1 standard deviation from the mean. (A) Cells irradiated with 254-nm UV light. Symbols: ○, h^- *ura4*; □, PS317; △, h^- *rad1-1 ura4*; ▽, PS32r. (B) Procedure and symbols as in panel A, but cells were plated on YPD plus caffeine. (C) Cells exposed to gamma radiation. Symbols correspond to those in panel A. (D) Procedure and symbols as in panel C, but cells were plated on YPD plus caffeine.

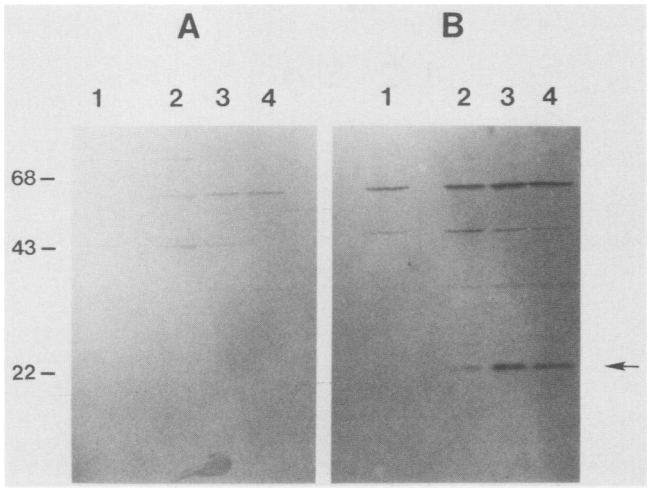

FIG. 8. Immunoblot of proteins from *E. coli* BL21 expressing the rad1 protein. Samples were electrophoresed on a 12% SDS-polyacrylamide gel. After transfer to nitrocellulose, the blot was incubated with rabbit antibodies diluted 1:100. Detection was with horseradish peroxidase-conjugated goat anti-rabbit antibodies. Sizes of molecular weight markers are given in kilodaltons on the left. (A) Probed with preimmune serum. Lanes: 1, immediately before addition of IPTG to a final concentration of 0.4 mM; 2 to 4, 30 (lane 2) 90 (lane 3), and 180 (lane 4) min after addition of IPTG. (B) Same samples, but blot was probed with serum from the same rabbit after immunization with keyhole limpet hemocyanin-conjugated peptide. Arrow indicates tentatively identified rad1 protein.

expressed to a protein product. We do not favor this interpretation, however, since (i) the two in-frame ATGs (at bp 804 and 815) have a very poor fit to the derived consensus for eucaryotic start codons (18) and (ii) this ORF does not score high in algorithms that identify protein-coding DNA (45). Rather, these findings show that an extended region of 5′-flanking noncoding sequence is needed for full expression. Specifically, the complementation-negative insertion at the *Spe*I site, at 151 bp upstream of the putative start codon and 61 bp upstream of the apparent TATA box, hints at the existence of controlling elements upstream of the promoter, a situation not unprecedented for *S. pombe* genes (39). In this context, one may note the presence at bp 440 of the sequence CCAAT, the consensus for the CAT box found in vertebrate promoters. The significance of this finding is unclear at this point since CAT elements have not been functionally defined in yeast promoters.

There are several indications that *rad1* is not a highly expressed gene. First, we have not been able to detect a *rad1* transcript by Northern (RNA) hybridization (data not shown). Also, with primer extension, only by the nonstandard technique of including label in the reverse transcriptase reaction could we obtain a signal for the 5′ end of the transcript. Second, repeated attempts to detect the rad1 protein by immunoprecipitation or by immunoblot analysis of extracts from *S. pombe* cells have been unsuccessful (data not shown). Third, a very low codon bias index for this gene indicates that the translation product is in low abundance. In *S. pombe*, not only is the codon bias of weakly expressed genes weaker than that of highly expressed genes as in most organisms, but there is a preference for codons other than those used in highly expressed genes (42). In *rad1*, in every multimembered codon family, the codon bias coincides with that of weakly expressed *S. pombe* genes (codon bias index = −0.07, calculated as described elsewhere [39]).

Some features of the deduced protein sequence of the *rad1* gene product are shared by the ubiquitin-conjugating enzymes of *Saccharomyces cerevisiae*, RAD6 and CDC34 (14, 17), such as low molecular weight, overall hydrophilic amino acid sequence, and particularly long stretches of acidic amino acid residues (mainly aspartate in RAD6 and CDC34) near the carboxy terminus (14, 38). In certain nuclear and nucleolar proteins from higher eucaryotes, such as nucleoplasmin and the high mobility group of proteins, acidic stretches, composed mainly of glutamate as in the rad1 protein, are also found. Computer-assisted similarity searches of these proteins and the rad1 protein, however, fail to reveal any significant additional amino acid sequence similarities, nor has any strong similarity been found between the rad1 protein and any other protein in the PIR data base (release 21.0, 30 June 1989). Likewise, *rad1* does not hybridize, even at low stringency, with any of the cloned *Saccharomyces cerevisiae* genes *RAD1* (36), *RAD2* (33), *RAD3* (32), *RAD6* (37), *RAD10* (47), and *RAD52* (1) (data not shown). On the other hand, under moderately stringent conditions, *Saccharomyces cerevisiae RAD6* does recognize fragments in *S. pombe* genomic DNA (data not shown). Thus, if there exists a counterpart to *Saccharomyces cerevisiae RAD6* in *S. pombe*, it is likely a gene other than *rad1*. The high negative net charge of this protein makes it unlikely to bind DNA directly. Rather, it could act by binding to other, positively charged proteins that in turn bind to DNA. Conceivably, it could have an early role in the series of events from detection of DNA damage to actual repair of it. Stretches of acidic residues near the carboxy terminus are also found in some transcriptional activators (35), raising the possibility that the *rad1* product acts by altering expression of other genes.

We have noted a grouping of leucine residues in the rad1 protein sequence that bears a superficial resemblance to the previously described leucine zipper (21). From residues 104 to 128, four leucines are found at regular intervals, but with a periodicity of 8 rather than 7. These four leucines are immediately followed by another two (134 and 140) with a periodicity of 6. The functional significance, if any, of this arrangement remains to be established.

The fact that the phenotypes studied of the constructed null allele of *rad1* are so closely similar to those of the initially isolated *rad1-1* allele strongly indicates that the mutation in *rad1-1* is one that completely destroys function of the protein.

Further studies on *rad1* will include purification of the protein expressed in *E. coli* or overexpressed in *S. pombe*, to allow characterization in vitro of its biochemical properties. Also, defined alterations of the chromosomal *rad1* locus as well as sequencing of the *rad1-1* allele will give insight into relationships between primary structure and function of the gene product. When the pattern of expression of this gene is defined, it will be possible to draw conclusions about what part *rad1* plays in the cellular response to DNA damage as well as in recombination.

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