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RAD3 Gene of *Saccharomyces cerevisiae*: Nucleotide Sequence of Wild-Type and Mutant Alleles, Transcript Mapping, and Aspects of Gene Regulation

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We determined the complete nucleotide sequence of the *RAD3* gene of *Saccharomyces cerevisiae*. The coding region of the gene contained 2,334 base pairs that could encode a protein with a calculated molecular weight of 89,796. Analysis of *RAD3* mRNA by Northern blots and by S1 nuclease mapping indicated that the transcript was approximately 2.5 kilobases and did not contain intervening sequences. Fusions between the *RAD3* gene and the *lac'Z* gene of *Escherichia coli* were constructed and used to demonstrate that the *RAD3* gene was not inducible by DNA damage caused by UV radiation or 4-nitroquinoline-1-oxide. Two UV-sensitive chromosomal mutant alleles of *RAD3*, *rad3-1* and *rad3-2*, were rescued by gap repair of a centromeric plasmid, and their sequences were determined. The *rad3-1* mutation changed a glutamic acid to lysine, and the *rad3-2* mutation changed a glycine to arginine. Previous studies have shown that disruption of the *RAD3* gene results in loss of an essential function and is associated with inviability of haploid cells. In the present experiments, plasmids carrying the *rad3-1* and *rad3-2* mutations were introduced into haploid cells containing a disrupted *RAD3* gene. These plasmids expressed the essential function of *RAD3* but not its DNA repair function. A 74-base-pair deletion at the 3′ end of the *RAD3* coding region or a fusion of this deletion to the *E. coli lac'Z* gene did not affect either function of *RAD3*.

In the yeast *Saccharomyces cerevisiae*, the incision of damaged DNA during excision repair requires at least five genes, designated *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10* (7). Mutations in these genes render cells abnormally sensitive to killing by UV radiation and by UV-mimetic compounds (24, 30). In addition, such mutants are defective in the excision of base damage caused by these agents, and in vivo DNA strand breaks normally associated with the early steps of excision repair cannot be detected (24, 30). To date, none of the proteins presumably encoded by these *RAD* genes has been isolated, and hence, the biochemistry of the excision repair of bulky base damage in yeasts has not been elucidated. Our aim was to understand the structure and function of the *RAD* genes required for incision of damaged DNA in *S. cerevisiae*, and we approached this goal by isolating and characterizing these genes with the intent of overproducing the proteins they encode.

In previous studies from this laboratory, a recombinant DNA plasmid containing the *RAD3* gene (pNF3000) was isolated from a yeast genomic library by complementation of the UV sensitivity of *rad3* mutants (16). Integration of an internal fragment of *RAD3* resulted in the disruption of the yeast chromosomal *RAD3* gene (17). Such disruptions are lethal in haploid cells and, together with the results of other experiments, demonstrate that, in addition to its requirement for the excision repair of base damage, the *RAD3* gene is essential for the viability of *S. cerevisiae* in the absence of DNA damage (17). Similar results have been reported by Higgins et al. (8). Existing *rad3* mutants are viable in the absence of DNA damage, suggesting that the excision repair and essential functions of the gene may be distinct, although other explanations for the presence of two phenotypes associated with mutations in one gene are tenable.

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At the present, the nature of the essential and DNA excision repair functions of *RAD3* are not known. To begin to address these and other questions concerning the structure and function of *RAD3*, we characterized the gene further. We report here the subcloning of the gene, its complete nucleotide sequence, and the mapping of the *RAD3* transcript. In addition, we rescued the *rad3-1* and *rad3-2* chromosomal alleles with plasmids and identified a mutant site in each by sequence analysis. These and other mutant plasmids were then introduced into a haploid strain with a disrupted *RAD3* gene to test for the viability function of the genes present on the plasmids. Finally, by measurement of β-galactosidase activity expressed in cells transformed with plasmids containing *RAD3-lac'Z* gene fusions, we also showed that the *RAD3* gene was not inducible by treatment of cells with UV radiation or 4-nitroquinoline-1-oxide.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* HB101 was used for the propagation of plasmids. The yeast haploid strains used have all been previously described (16, 19). The plasmids used in this study are listed in Table 1. A diploid strain *rad3-1/rad3*^{0}*:TRP1 ura3-52/ura3-52 Δtrp1/Δtrp1 lys2-801/lys2-801 ade2-101/ade2-101 Δhis3/Δhis3 can*^{S}*/CAN*^{R} was constructed to test plasmids for their ability to complement the essential function of *RAD3*. This diploid contains (i) a nonlethal mutation of *RAD3* (*rad3-1*) that confers the phenotype of recessive UV sensitivity and of dominant viability and (ii) a *RAD3* disruption (*rad3*^{0}) that confers both UV sensitivity and recessive lethality to the diploid. This disruption (*rad3*^{0}*:TRP1*) was generated by integration of a plasmid carrying the *TRP1* gene and a *Bam*HI-*Bgl*II internal fragment of *RAD3*.

TABLE 1. Plasmids

Plasmid	Essential features	Reference
pNF3000	2μm origin, 6-kb *RAD3* insert	16
pNF3001	Centromeric vector, *Eco*RI-*Sal*I *RAD3* insert	This study
pNF3001-1	Same as pNF3001 but contains an *rad3-1* mutation	This study
pNF30001-2	Same as pNF3001 but contains an *rad3-2* mutation	This study
pNF3005	2μm origin, 3.2-kb *RAD3* insert deleted of 3′ end	This study
pNF3006	pNF3005 with *lac′Z* gene fused at *Bam*HI site	This study
pNF3008	pNF3005 with *Xho*I-*Sal*I deletion	This study
pNF3009	pNF3008 with *lac′Z* gene fused at *Sal*I site	This study
pNF3515	pNF3000 with *lac′Z* gene fused at *Bam*HI site	This study
pNF3517	Integrating plasmid carrying insert from pNF3515	This study
pNF2	2μm-based vector, derivative of YEp24	18
pNF3	Integrating vector, derivative of pNF2	18
pMC931	Contains *lac′Z* gene used for fusions at *Bam*HI site	4
pMCRF31	Contains *lac′Z* gene used for fusions at *Sal*I site	G. Robinson and E. C. Friedberg, unpublished data

Culture media. YPD medium and minimal medium (supplemented with nutrients as required) were used for growth of *S. cerevisiae* as previously described (16). L broth was used for growth of *E. coli* and was supplemented with ampicillin (50 μg/ml) or kanamycin (15 μg/ml) as appropriate.

Preparation of DNA and transformation of cells. Plasmid DNA was isolated from *E. coli* by boiling of cells (10) followed by phenol extraction of the supernatant and cesium chloride-ethidium bromide centrifugation. Transformation of bacterial cells was performed as described by Okayama and Berg (21). Transformation of yeast cells was performed with spheroplasts generated with glusulase (9) or by treatment with lithium acetate (11).

Subcloning of the *RAD3* gene. The plasmid pNF3000 (16) was partially digested with *Sau*3A, and the DNA fragments were ligated into the vector pNF2 (18), which had been treated with *Bam*HI and calf alkaline phosphatase. The mixture was transformed into *E. coli* HB101, and cells resistant to kanamycin were selected and grown in liquid culture. DNA was extracted from *E. coli* transformants and used to transform a yeast *rad3-2* strain. Four yeast colonies resistant to UV radiation were identified, one of which (pNF3005) was shown to contain a plasmid with an insert of ~3.2 kilobases (kb).

DNA sequence determination. Restriction sites in the 3.2-kb fragment containing the *RAD3* gene were used to generate fragments that were cloned into M13mp10 and M13mp11 vectors (20) for DNA sequence determination by the chain termination method (28), using dideoxynucleotides and [α-^{35}S]dATP (3). Typically, between 300 and 400 bases could be read from three loadings on a 40-cm sequencing gel. The 3.2-kb DNA fragment was sonicated, blunt ended with S1 nuclease, and cloned into the *Hin*cII site of M13mp10 to generate random subclones. Initial DNA sequence determination revealed an open reading frame without a stop codon; hence, additional fragments derived from a 1.3-kb *Eco*RI fragment adjacent to the 3.2-kb fragment in pNF3000 (16) were also cloned, and their sequences were determined. All restriction sites used for cloning were crossed, the sequences of all but 40 base pairs (bp) of the open reading frame were determined for both DNA strands.

Isolation and sequence analysis of DNA fragments containing the *rad3-1* and *rad3-2* mutations. Plasmids containing the *rad3-1* and *rad3-2* mutations were isolated by a modification of the mutational rescue procedure (22) in which gaps in the centromeric plasmid pNF3001 were created by digestion with either *Hin*dIII and *Bam*HI, *Cla*I and *Bam*HI, *Cla*I and *Xba*I, or *Cla*I alone. After transformation of *rad3-1* and *rad3-2* strains with the gapped, linearized plasmids, colonies were screened for UV sensitivity. Plasmids pNF3001-1 and pNF3001-2 (Table 1) were isolated from UV-sensitive colonies (see Fig. 7) and propagated in *E. coli*, and the DNA fragments of interest were cloned into M13 for DNA sequence determination as described above.

Construction of *RAD3-lac′Z* gene fusions and measurement of their expression. The *Bam*HI fragment from plasmid pMC931 containing most of the coding region of the *E. coli lac′Z* gene (4) was cloned into the *Bam*HI sites of plasmids pNF3000 and pNF3005 in both orientations, generating the productive fusion plasmids pNF3515 and pNF3006 (Fig. 1). The gene fusion in pNF3515 was subcloned into the integrating vector pNF3 to generate pNF3517 (Fig. 1). Plasmid pNF3005 was cut with *Xho*I and partially digested with *Sal*I. Linear molecules were isolated, self-ligated, and transformed into *E. coli* to yield plasmid pNF3008 (Fig. 1). A 6.2-kb fragment of the *E. coli lac′Z* gene present in plasmid pMCRF31 (G. Robinson and E. C. Friedberg, unpublished data) was cloned into the single *Sal*I site of pNF3008 to yield the fusion plasmid pNF3009 (Fig. 1).

Quantitative expression of *E. coli* β-galactosidase was measured in liquid cultures of *E. coli* or yeast transformants by using a permeabilized cell assay with orthonitrophenyl-β-D-galactopyranoside as a substrate (25). Units of activity are defined as $1{,}000 \times OD_{420}/\text{time} \times \text{volume} \times OD_{600}$, where OD_{420} and OD_{600} are the optical densities at 420 and 600 nm, respectively (25). Qualitative measurement of β-galactosidase in plate cultures was carried out by transferring cells from agar plates to filter paper disks. The cells were lysed by immersing the disks in liquid nitrogen for several minutes, and β-galactosidase activity was detected by adding 50 μl of 2% XGal in 1.0 ml of Z buffer (25) to the filters and incubating them at 37°C.

Measurement of *RAD3* mRNA and transcript mapping. To identify *RAD3* mRNA, yeast cells from strain S288C were grown to an OD_{600} of ca. 1.0 in YPD medium, harvested by centrifugation, and suspended in a solution of 0.5 M NaCl, 0.2 M Tris-hydrochloride (pH 7.5), 0.01 M EDTA, and 1% sodium dodecyl sulfate. The cells were disrupted by vortexing with 0.3-mm glass beads in phenol-chloroform. The aqueous phase was reextracted with phenol-chloroform, and nucleic acids were precipitated with 70% ethanol (12). Nucleic acids were treated with glyoxal, fractionated on agarose gels by electrophoresis, transferred to nitrocellulose, and hybridized as previously described (19) with a radiolabeled plasmid containing a 1.5-kb internal fragment of *RAD3* and the yeast *URA3* gene.

The Berk and Sharp procedure (2) was used to determine the location of the 5′ and 3′ ends of the *RAD3* transcript. DNA-RNA hybridization was in 80% formamide at 45°C for 3 h, and subsequent S1 nuclease digestion (1,500 U) was

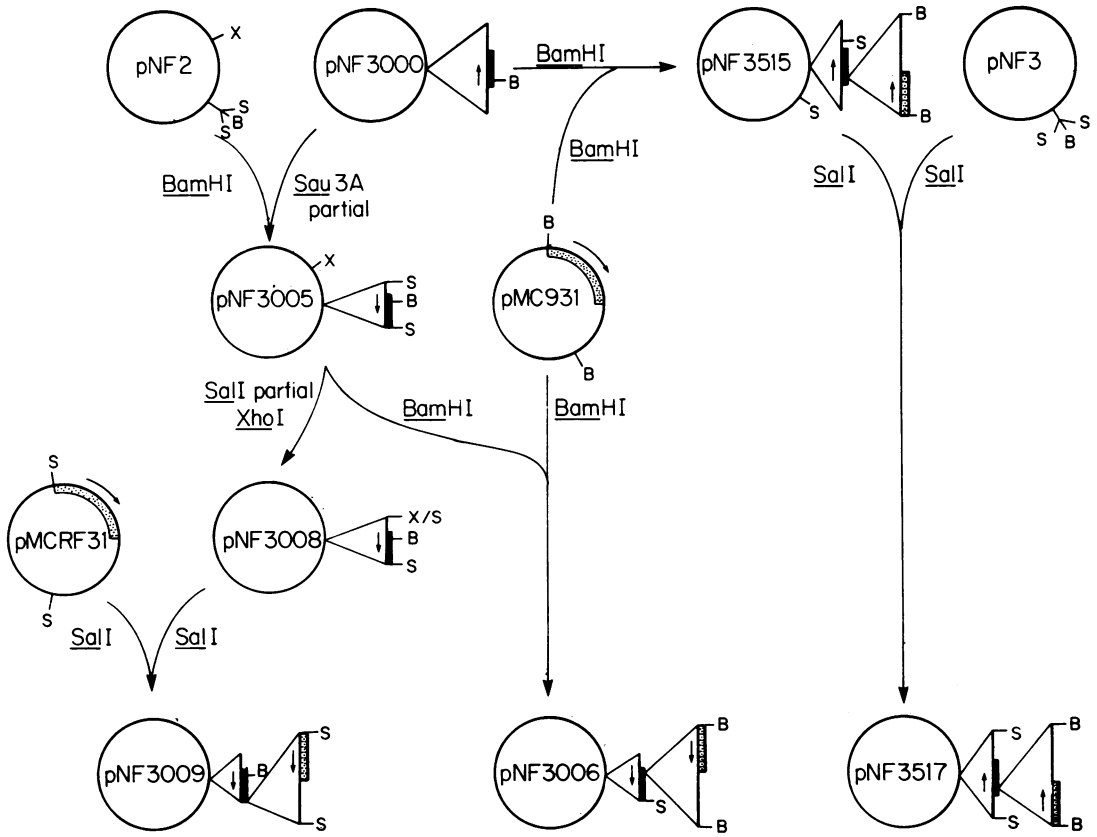


FIG. 1. Construction of plasmids. The origins of most of the fusion plasmids used in this study are shown schematically. Symbols: ■, *RAD3* sequences; ▩, *E. coli lac'Z* sequences. Arrows show the direction of transcription of the *RAD3* and *lac'Z* genes in each of the constructions. Restriction sites: S, *Sal*I; B, *Bam*HI; X, *Xho*I.

carried out at 37°C for 30 min. The 5′ end of the *RAD3* transcript was mapped by using a 5′ end-labeled 2.8-kb *Sal*I-*Eco*RI fragment from pNF3005. The 3′ end of the transcript was mapped by using the *Eco*RI-*Sal*I fragment from pNF3001. Agarose gels (1 to 2%) were run under both neutral (40 mM Tris-acetate [pH 7.5], 3 mM EDTA) and alkaline denaturing (30 mM NaOH, 1 mM EDTA) conditions.

RESULTS

Subcloning the *RAD3* gene. The *RAD3* gene was originally isolated on a ~6-kb yeast DNA insert (15). To localize the

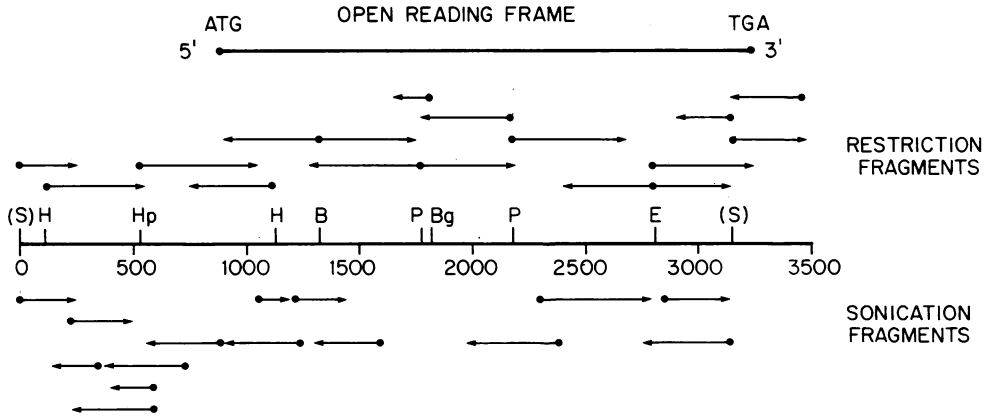


FIG. 2. Sequence determination strategy for the *RAD3* gene. The restriction map of the insert in pNF3005 extended between the two sites labeled S. Known restriction fragments and random fragments generated by sonication of the 3.2-kb insert were cloned into M13, and the sequences were determined. The start (●) and direction and termination (arrows) of the sequence for a particular clone are as indicated. Only restriction enzyme sites used to generate the restriction fragments for sequence determination are shown: S, = *Sal*I sites derived from cloning into pNF2; H, *Hin*dIII; Hp, *Hpa*I; B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; E, *Eco*RI.

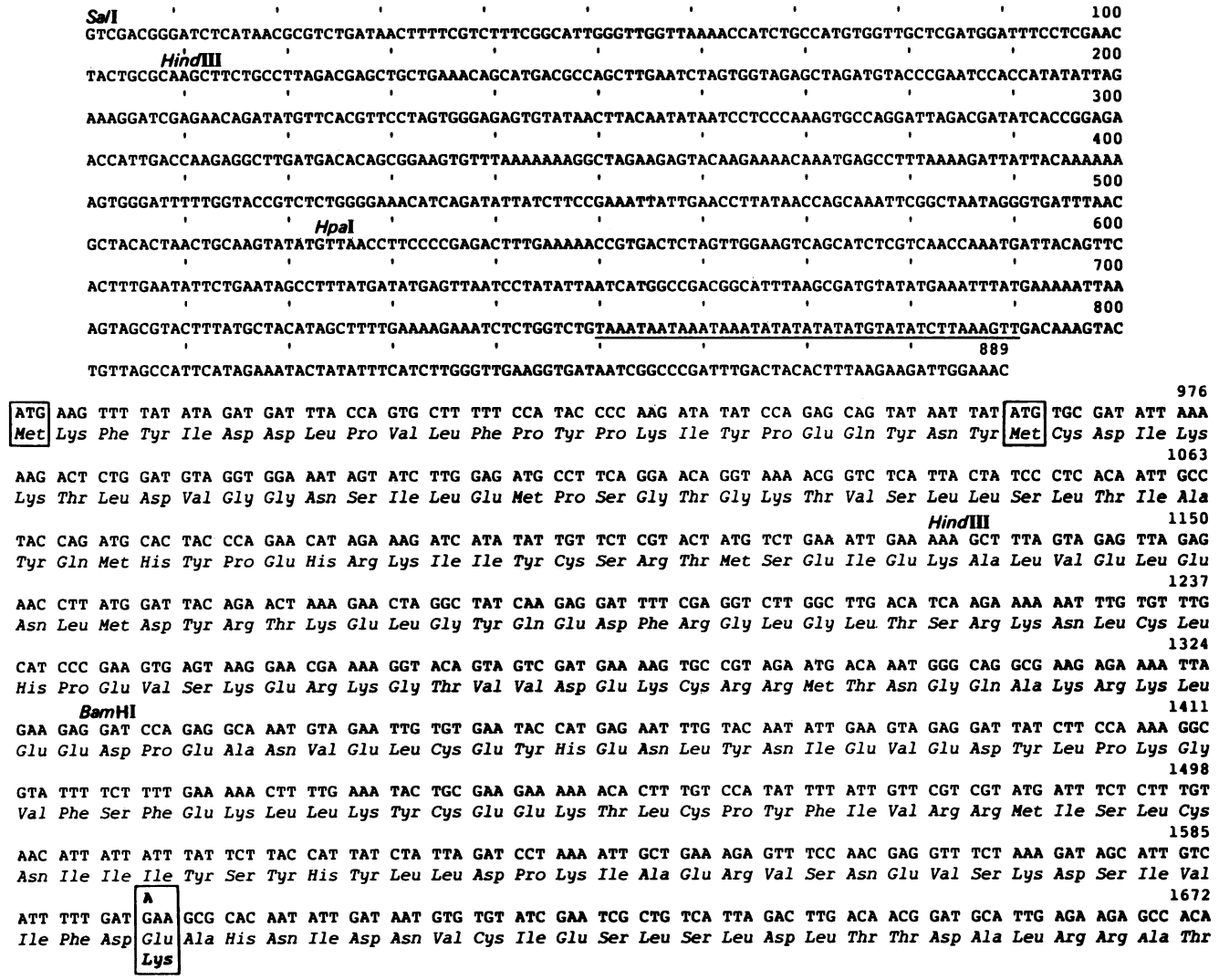


Figure 3 continued on next page

gene more precisely, plasmid pNF3000 was treated with *Sau*3A to generate a partial digest. This digest was ligated into plasmid pNF2, a vector derived from the multicopy autonomously replicating plasmid YEp24, and specifically designed for the cloning of *Sau*3A fragments into a unique *Bam*HI site and their retrieval by adjacent *Sal*I sites (18). Yeast *rad3* transformants were screened for enhanced resistance to UV radiation, 3kand several distinct recombinant plasmids were identified. Restriction analysis of these showed that the smallest DNA insert was ~3.2-kb, and this plasmid (pNF3005) (Fig. 1) was selected for further study.

Nucleotide sequence of *RAD3*. The 3.2-kb insert in pNF3005 was mapped, and a DNA sequence determination strategy was devised (Fig. 2). Analysis of the sequence revealed an open reading frame without a termination codon, suggesting that the 3.2-kb fragment does not contain the entire *RAD3* gene. Further subcloning and DNA sequence determination was carried out with plasmid pNF3205, which contains a 1.3-kb *Eco*RI fragment immediately 3′ to the fragment present in pNF3005 (fragment E [16]). The open reading frame continued in this fragment and a TGA translational stop codon was identified as beginning 74 nucleotides downstream from the end of the 3.2-kb fragment originally sequenced.

The entire sequenced region comprised 3,490 bp (Fig. 3), 2,334 of which were in a single open reading frame beginning at position 890 in the sequence and terminating at position 3,223. The sequence around the first ATG codon contained an A at position −3 and a purine (A) at position +4. Hence, the heptanucleotide ANNAUGA present in the RNA was that typically encountered at the translational start site in eucaryotic mRNAs (13). The next ATG in the same reading frame (beginning at position 959 of the sequence) was part of the heptanucleotide YNNAUGY (where Y is a pyrimidine), typical of internal AUG codons in eucaryotic mRNAs (13). The open reading frame contained 778 codons and could be translated to yield a polypeptide of 89,796 daltons, assuming that the first ATG initiated translation.

There was no perfect TATA box 5′ to the transcriptional start site; however, the region between positions 750 and 790 was extremely A+T rich (37 of 40 nucleotides were A or T) (Fig. 3) and contained sequences that strongly resembled a TATA box. A consensus AATAAA polyadenylation signal (23) was not present at the 3′ end; however, the closely related sequences TATAAA, ATTAAA, and ACTAAA were identified at 19, 44, and 97 nucleotides, respectively, downstream from the TGA stop codon (Fig. 3). The sequence between positions 3,409 and 3,480 bore a strong resemblance

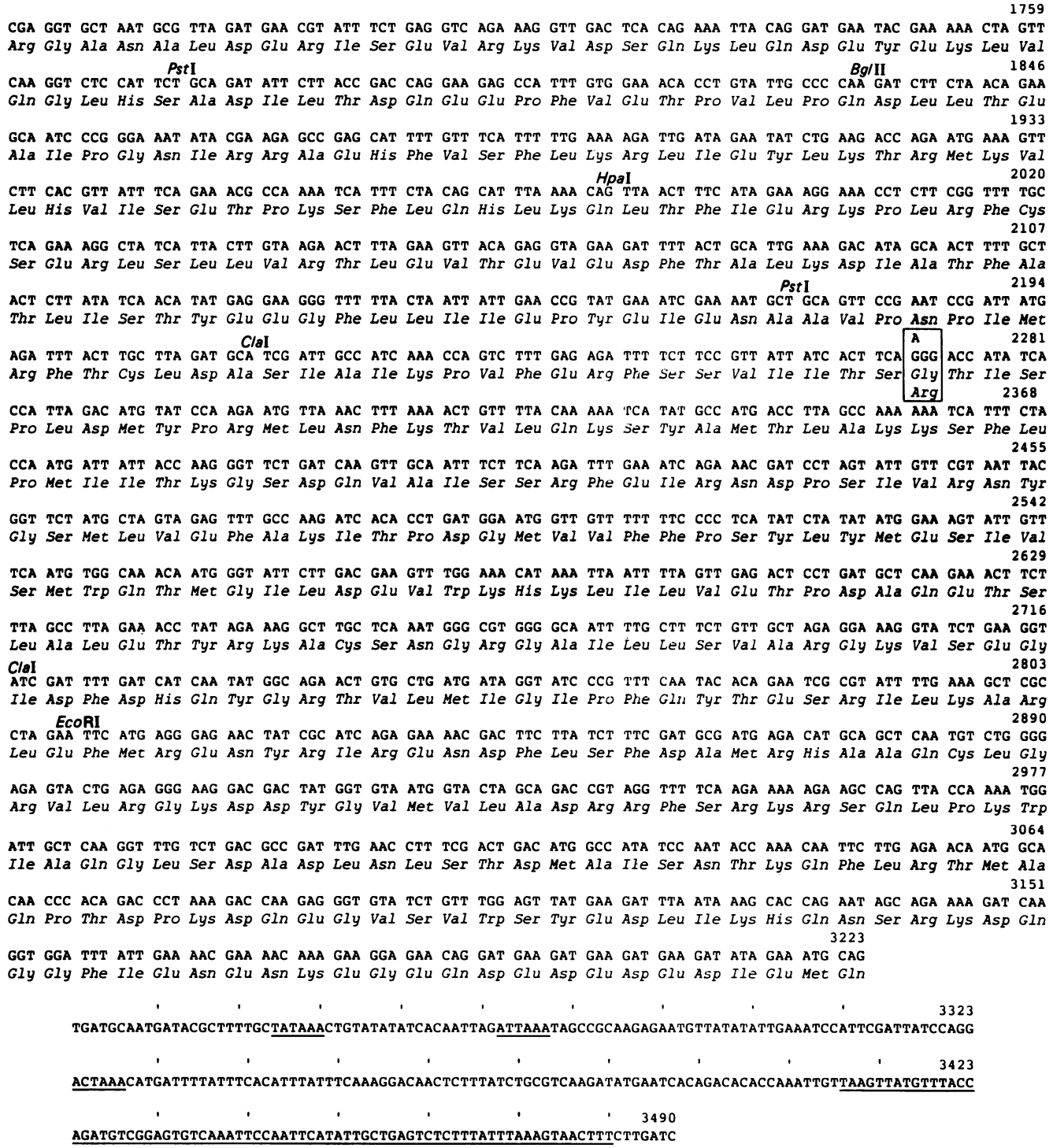


FIG. 3. Nucleotide sequence and predicted amino acid sequence of the *RAD3* gene. The DNA sequence is numbered from nucleotides 1 to 3,490. The AtT-rich region between nucleotides 750 and 790 is underlined. The first and second ATGs in the open reading frame that starts at position 890 are boxed. The open reading frame is translated in the standard three-letter amino acid code. Sequences that resemble polyadenylation sites are underlined, as is the extended sequence that may be involved in transcript termination. The mutations in the *rad3-1* and *rad3-2* alleles are boxed and are shown at the nucleotide and amino acid levels. Relevant restriction sites are also shown.

to the consensus sequence TAG. . .TATGT. . . .(A+T rich). . . .TTT. . . . . , which may play a role in transcription termination in yeasts (33). We did not detect the internally conserved sequence TACTAAC, which is associated with all studied RNA polymerase II-transcribed yeast genes known to contain introns (14).

A computer search revealed no significant homology at either the nucleotide or amino acid levels with any other sequences in the data base surveyed. The *RAD3* coding sequence also showed no significant homology with that of the *RAD1* gene of *S. cerevisiae* (31) nor with that of the *uvrC* gene of *E. coli* (27).

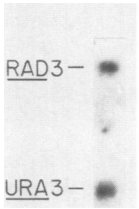


FIG. 4. Northern blot analysis of *RAD3* RNA. Total nucleic acid (25 μg) isolated from strain S288C was glyoxylated, electrophoresed on an agarose gel, and transferred to nitrocellulose. The blot was probed with a nick-translated plasmid (5×10^7 cpm/μg) containing the *RAD3* and *URA3* genes. The positions of the transcripts are indicated.

***RAD3* transcript.** DNA-RNA hybridization by the Northern technique revealed a single RNA species of ~2.4 kb that hybridized to an internal fragment of the *RAD3* gene (Fig. 4). Relative to the amount of *URA3* transcript detected in control hybridizations, we estimate that there are <5 copies of *RAD3* mRNA per cell in exponentially growing populations of *S. cerevisiae* (assuming that the *URA3* transcript is present at about 5 to 10 copies per cell [M. Rose, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1982]). To map the ends of the mRNA, DNA probes specific for either the 5′ or 3′ end of the *RAD3* gene were hybridized to total and to polyadenylic acid-selected yeast RNA, and the hybrids were digested with S1 nuclease. After agarose gel electrophoresis, a single labeled band was detected in each case. The RNA that hybridized to the 5′-labeled DNA probe (Fig. 5) was polyadenylated and extended 2,350 ± 50 bp in the 5′ direction from position 3,151 in the sequence. The RNA that hybridized to the 3′-labeled DNA probe (data not shown) extended to position 3,350 ± 30 bp in the sequence. The size of the S1 nuclease-protected fragment in each case was identical under neutral and alkaline conditions of electrophoresis (Fig. 5). Based on these results, we concluded that the *RAD3* gene had no intervening sequences and that transcription of the gene initiated at approximately position 800 in the sequence, between the A+T-rich region spanning nucleotides 750 to 790 and the first in frame ATG at nucleotide 890 (Fig. 3). Transcription terminated approximately 127 nucleotides 3′ to the TGA stop codon, which was located at nucleotide 3,223 (Fig. 3). The size of the *RAD3* transcript estimated from the S1 nuclease mapping data (~2.5 kb without the polyadenylate tail) was in good agreement with that measured by Northern analysis (~2.4 kb) (see above). Higgins et al. (8) have determined a similar size for the message.

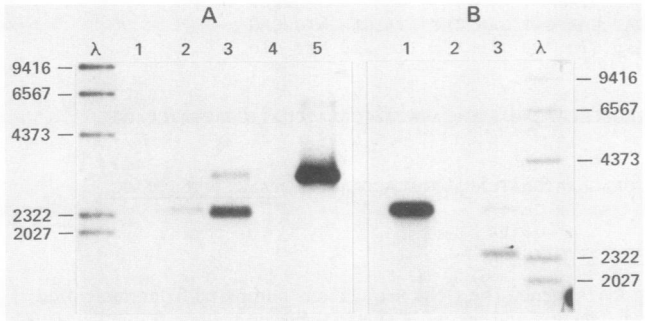


FIG. 5. Mapping the 5′ end of the *RAD3* transcript. RNA was isolated from strain S288C, hybridized to 5′ end-labeled DNA, treated with S1 nuclease, and electrophoresed on neutral (A) or alkaline (B) agarose gels. λ is λ DNA restricted with *Hin*dIII to generate marker fragments with the sizes (nucleotides) indicated. (A) Lanes 1, probe with nonpolyadenylated RNA; 2, probe with polyadenylated RNA; 3, probe with total RNA; 4, probe with no RNA; 5, probe with no S1 nuclease treatment. (B) Lanes: 1, probe with no S1 nuclease treatment; 2, probe with no RNA; 3, probe with polyadenylated RNA.

Regulation of *RAD3* expression. Plasmids with in-frame fusions between the *RAD3* and *E. coli lac′Z* (4) genes expressed detectable β-galactosidase activity in *E. coli* transformants regardless of the orientation of the *lac′Z* fragment. However, for each of the two orientations of *lac′Z*, only one of the plasmids expressed β-galactosidase activity in *S. cerevisiae* (Fig. 1 and Table 2). This result was consistent with *RAD3* sequencing data, which predicted that the *lac′Z* gene would be both in the correct transcriptional orientation and correct translational reading frame in only one plasmid. The amount of β-galactosidase activity expressed in cultures of cells transformed with the multicopy fusion plasmid pNF3515 was about 15 times greater than that expressed in cultures transformed with the integrating plasmid pNF3517 containing the identical gene fusion (Table 2). The amount of β-galactosidase detected also varied with the particular gene fusion carried on the same multicopy vector. Thus, cells transformed with plasmid pNF3009 consistently contained about twice the amount of β-galactosidase present in cells transformed with pNF3515 (Table 2), and the latter contained about three times the activity present in cells transformed with pNF3005 (Table 2). The reasons for these differences are not known. They do not bear any obvious relationship to the position of the fusion relative to the 5′ end of the gene and hence may simply reflect plasmid copy number effects or varying stability of β-galactosidase expressed from different gene fusions. We consistently observed that for all *RAD3-lac′Z* gene fusions examined, the amount of β-galactosidase activity was about twice as high in cultures of haploid transformants as in cultures containing equivalent numbers of diploid transformants (Table 2). The same result was observed with a plasmid containing a *HIS3-lac′Z* fusion and may represent a difference in plasmid copy number between haploid and diploid cells.

DNA damage-inducible (*DIN*) genes have been isolated from *S. cerevisiae* (25), but it is not known whether any of these are *RAD* genes involved in excision repair. To investigate the possible inducible expression of the *RAD3* gene in yeast cells treated with DNA-damaging agents, we used *RAD3-lac′Z* gene fusions present on either multicopy (pNF3515) or integrating (pNF3517) plasmids (Fig. 1). *RAD*

TABLE 2. Expression of *RAD3* functions and β-galactosidase activity from fusion plasmids

Plasmid	Presence (+) or absence (−) of *RAD3* function:		β-Galactosidase activity (U)	
	Excision repair	Viability	Haploid	Diploid
pNF3000	+	+		
pNF3001	+	+		
pNF3001-1	−	+		
pNF3001-2	−	+		
pNF3005	+	+	6	4.5
pNF3006	−	−		
pNF3008	+	+		
pNF3009	+	+	34.7	16.4
pNF3515	−	−	17.5	9.1
pNF3517	ND[a]	ND	1.1	ND

[a] ND, Not determined.

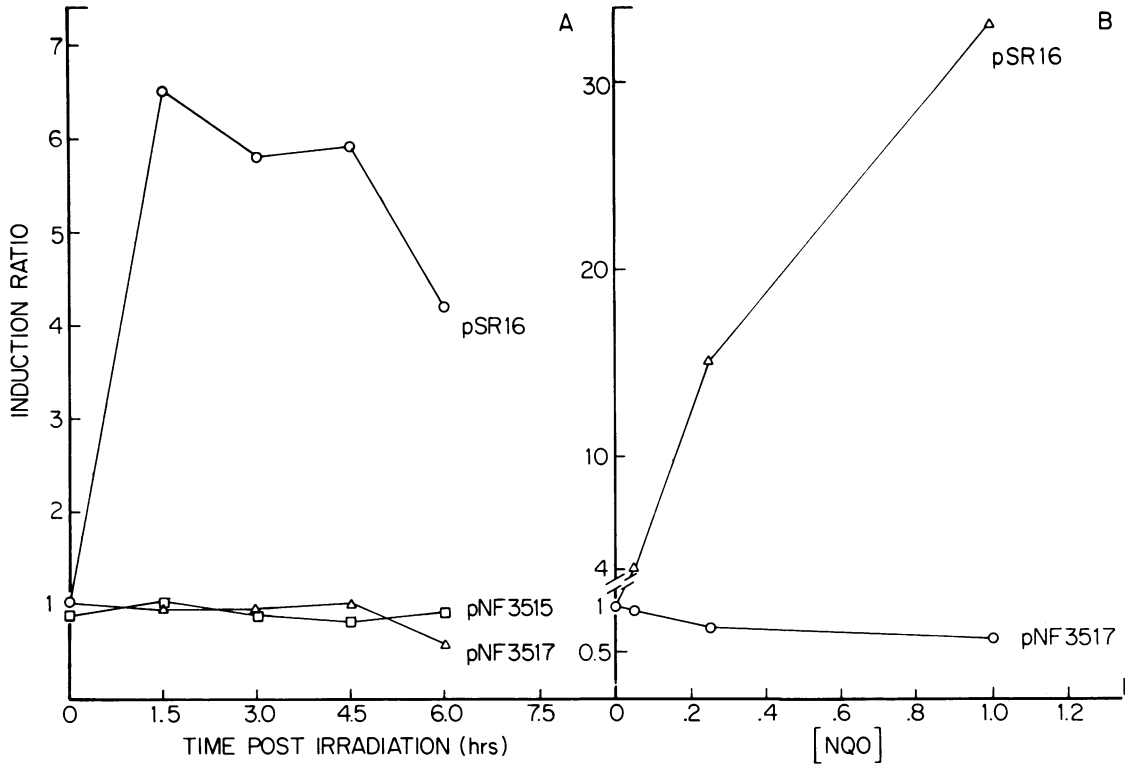
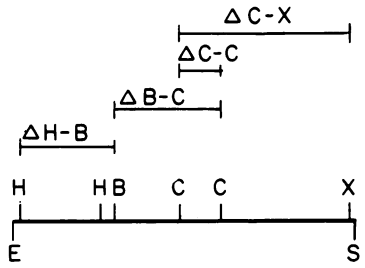


FIG. 6. Expression of *RAD3-lac'Z* fusion activity under conditions of DNA damage. Yeast strains containing *RAD3-lac'Z* fusions or a *DIN1-lac'Z* fusion were (A) UV irradiated or (B) treated with 4-nitroquinoline-1-oxide (the concentration is shown in micrograms per milliliter), and β-galactosidase activity was measured by using orthonitrophenyl-β-D-galactopyranoside as the substrate. The induction ratio is the β-galactosidase activity measured in treated cultures divided by that measured in untreated control cultures.

transformants were grown in culture and exposed to UV radiation at an incident dose of 20 J/m^2. Cell growth was continued, and β-galactosidase activity was measured at various times. The induction ratio, expressed as the amount of β-galactosidase activity in treated compared with untreated control cultures was generally slightly less than one (Fig. 6A). A *DIN1-lac'Z* fusion (pSR16) from *S. cerevisiae* (25), known to be inducible by DNA damage, yielded an induction ratio of 6.5 under these experimental conditions (Fig. 6A). Treatment of pSR16-transformed cells with 4-nitroquinoline-1-oxide resulted in a 33-fold increase in the level of β-galactosidase (Fig. 6B). However, no increase was detected in treated cells transformed with the integrating plasmid pNF3517 containing the *RAD3-lac'Z* fusion (Fig. 6B). In fact, as was the case after treatment with UV radiation (Fig. 6a), there was a slight decrease in β-galactosidase activity after exposure to 4-nitroquinoline-1-oxide. Based on these results, we concluded that the *RAD3* promoter was not induced by DNA damage caused by UV radiation or 4-nitroquinoline-1-oxide.

Analysis of *RAD3* DNA repair and essential functions. As indicated previously, the *RAD3* gene of *S. cerevisiae* was required both for the excision repair of DNA damage and for the viability of haploid cells in the absence of DNA damage. To gain some insight into the regions of the gene that encode these two functions, we determined the precise positions of the mutation in two *rad3* mutants. The *rad3-1* and *rad3-2* chromosomal mutant alleles were rescued by transforming mutant *rad3* strains with linear plasmids containing defined gaps (22) created by digestion of the centromeric plasmid pNF3001 with selected restriction enzymes (Fig. 7). The



UV^S COLONIES FOLLOWING TRANSFORMATION OF

	rad3-1	rad3-2
ΔH-B	16/31 (52%)	7/34 (21%)
ΔB-C	28/28 (100%)	30/30 (100%)
ΔC-C	9/24 (38%)	21/21 (100%)
ΔC-X	3/13 (23%)	30/30 (100%)

FIG. 7. Mapping and marker rescue of the *rad3-1* and *rad3-2* chromosomal alleles. The *Eco*RI-*Sal*I insert of pNF3001 is shown. Gaps (sizes of which are indicated by the deletions [Δ] shown) were constructed by treatment of the plasmid with the indicated restriction enzymes (22). Gapped plasmids were then transformed into *rad3-1* and *rad3-2* mutants, and the resulting colonies were scored for sensitivity to UV light (UV^s). The numbers show the frequency of UV^s colonies after transformation with each gapped (deleted) plasmid. Restriction sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; C, *Cla*I; X, *Xho*I; S, *Sal*I.

rad3-1 mutation was localized to an 885-bp *Bam*HI-*Cla*I fragment (Fig. 7), and the *rad3-2* mutation was localized to a 502-bp *Cla*I fragment (Fig. 7). The plasmids containing these fragments (pNF3001-1 and pNF3001-2, respectively) were isolated from *S. cerevisiae*, propagated in *E. coli*, and cloned into phage M13. Sequence analysis of the *Bam*HI-*Cla*I fragment containing *rad3-1* identified a G · C→A · T transition mutation at position 1,595 in the sequence, resulting in replacement of glutamine acid with lysine at codon 236 in the open reading frame (Fig. 3). Sequence analysis of a *Pst*I-*Eco*RI fragment with the *rad3-2* mutation identified a G · C→A · T transition at position 2,270, resulting in replacement of glycine with arginine at codon 461 (Fig. 3). As a control to investigate whether these or other mutations might have arisen during the rescue procedure, we determined the sequences of other regions of pNF3001-1 and pNF3001-2. No alterations were found in these regions. We noted that a number of the gapped plasmids which did not span the site of the *rad3-1* or *rad3-2* mutations nonetheless rescued these markers (Fig. 7), possibly because of a gap extention in vivo or gene conversion events (22).

The *rad3-1* and *rad3-2* strains are viable under normal growth conditions. However, since the mutant alleles were rescued from the yeast genome in which other possible mutations (either in *RAD3* or in some other gene) may have contributed to the phenotype, we determined whether these plasmids retained the essential function of the *RAD3* gene. To do this, it was necessary to construct a specific diploid yeast strain. This diploid contained an insertional mutation in one of the *RAD3* genes which inactivated both the excision repair and essential functions and which was linked to a *TRP1* gene for selection. The other *RAD3* gene carried a point mutation that inactivated only the excision repair phenotype (Fig. 8; see above). When this diploid strain (*rad3*^{0}/*rad3-1*) was transformed with a plasmid that did not contain the *RAD3* gene, it was UV sensitive, but viability was maintained by the *rad3-1* allele. Sporulation followed by tetrad analysis showed a 2^+:2^0 segregation of viability (Fig. 8). On the other hand, when the diploid was transformed with a plasmid that contains a wild-type *RAD3* gene, it was UV resistant, and tetrad analysis showed 4^+:0^0, 3^+:1^0, and 2^+:2^0 segregation of viability (Fig. 8). In these cases, some of the viable haploids were *TRP*$^+$ and consequently *rad3*^{0}, but were dependent for their viability on the presence of the plasmid carrying *RAD3*. Loss of this plasmid resulted in the formation of microcolonies which ceased growth after a few divisions. By this analysis, we demonstrated that plasmids pNF3001-1 and pNF3001-2 derived by rescue of the *rad3-1* and *rad3-2* chromosomal mutations, respectively, retained the essential function of *RAD3* and, as expected, did not complement the UV sensitivity of the diploid or of *rad3* haploid mutants (Table 2).

The *RAD3-lac'Z* fusion plasmids and a plasmid missing the 3′ end of the *RAD3* gene were also tested by this procedure. Transformation of the *rad3*-1/*rad3*^{0} diploid with the fusion plasmids pNF3515 and pNF3006 (Fig. 1) did not result in complementation of the UV sensitivity of this strain (Table 2), and tetrad analysis showed 2^+:2^0 segregation of viability with no viable *TRP*$^+$ haploids. Thus, fusion of the *lac'Z* gene at the *Bam*HI in the *RAD3* gene inactivated both the excision repair and essential functions of *RAD3* (Table 2). Plasmid pNF3009 containing a fusion of the *lac'Z* gene at the *Sal*I site near the 3′ end of the *RAD3* gene (Fig. 1), as well as the nonfusion plasmid pNF3005 (Fig. 1), both were missing 74 nucleotides from the 3′-terminal coding region of

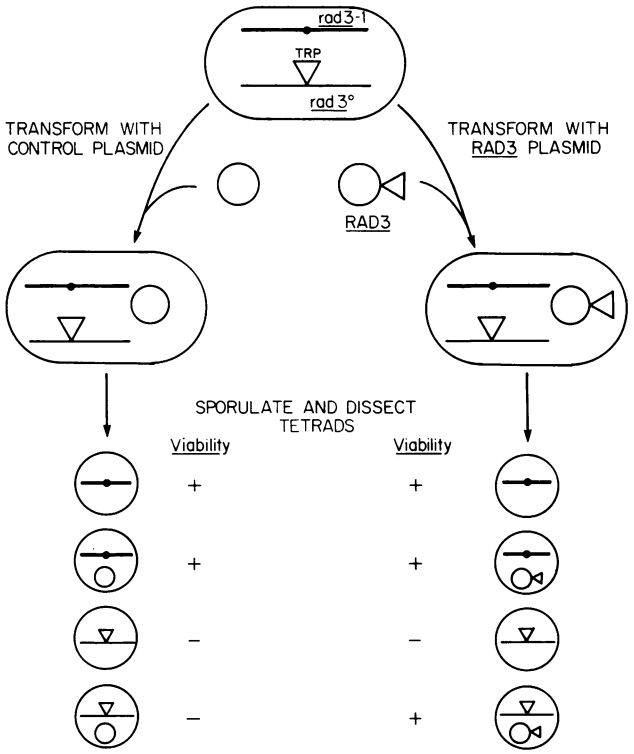


FIG. 8. Use of a *rad3*/*rad3*^{0} strain for analysis of the *RAD3* essential function. Transformation of a *rad3*/*rad3*^{0} strain with a plasmid not carrying a viability-proficient *RAD3* gene followed by sporulation and tetrad analysis leads to a 2^+:2^0 segregation of viability (left). If the transforming plasmid carries the essential function, then some tetrads give 3^+:1^0 (right) or 4^+:0^0 (not shown) segregation of viability. *TRP*$^+$ *rad3*^{0} haploids whose viability is dependent on the continued maintenance of the plasmid can thus be isolated.

RAD3. However, both plasmids complemented the UV sensitivity of *rad3* mutants (Table 2). In addition, after transformation and sporulation of the *rad3*-1/*rad3*^{0} diploid, we were able to isolate haploid *rad3*^{0} *TRP*$^+$ cells whose viability was dependent on the presence of either plasmid. Thus, these plasmids also complemented the essential function of the *RAD3* gene (Table 2).

DISCUSSION

Subcloning of a 6-kb DNA fragment containing the yeast *RAD3* gene localized the gene to a smaller fragment of 3.2 kb. DNA sequence determination revealed a long open reading frame which continued for 74 nucleotides into an adjacent fragment. The entire open reading frame was 2,334 bp and contained 778 codons that could encode a protein with a molecular weight calculated at 89,796. The ATG triplet that marked the beginning of the open reading frame was presumed to be the translation initiation codon of the *RAD3* gene since it was the first start codon downstream from the position at which the 5′ end of the *RAD3* transcript was mapped. Furthermore, a heptanucleotide sequence that included this ATG triplet is prototypic of the great majority of eucaryotic translational start codons, whereas one that includes the next ATG is typical of an internal methionine codon. In addition, positioning of the yeast *GAL1* promoter immediately 5′ to the first ATG codon in the open reading frame generated a plasmid that fully complements the UV sensitivity of *rad3* mutants when the transformants are

grown in the presence of galactose, but not in the presence of dextrose (Naumovski and Friedberg, unpublished data).

As is often the case with yeast promoters, no consensus TATA box was found; however, the 5′ noncoding sequences revealed an AT-rich region upstream of the transcriptional start that may constitute a functional promoter. A consensus polyadenylation signal was not present in the 164-bp 3′ to the translational stop codon, but several closely related sequences were identified. In addition, 3′ noncoding sequences closely resembling those thought to be important in transcriptional termination in a number of other yeast genes were noted. Neither the nucleotide sequence nor the predicted amino acid sequence of *RAD3* were homologous with other genes for which such information is available. It may be of interest that the estimated size of RAD3 protein is similar to that of UvrB protein (Mr, ~84,000), which is required for the excision repair of bulky base damage in *E. coli* (26). The nucleotide and amino acid sequences of the coding region of the *uvrB* gene are not available for comparison with those of *RAD3*; however, in previous studies (16), pNF3000 did not complement the UV sensitivity of a *uvrB* strain.

The *RAD3* transcript appears to be constitutively present at a level of less than five copies per cell, and there is no evidence that expression of the gene is enhanced in yeast cells exposed to DNA damage by UV radiation or by 4-nitroquinoline-1-oxide. This observation provides an interesting contrast to the situation in *E. coli* in which it has been shown that the *uvrA*, *uvrB*, and *uvrC* (26, 29, 32) genes, all of which play a role in excision repair of DNA in vivo, are inducible by DNA damage (1, 26). Studies are currently in progress in this laboratory to determine whether other genes in the *RAD3* epistasis group are inducible by DNA damage.

Previous studies (8, 17) have shown that *rad3* mutants can have two distinct phenotypes. Some mutants are UV sensitive but are perfectly viable under normal conditions that preclude DNA damage. One possible explanation for these results is that the RAD3 protein has two distinct biochemical functions, one of which is involved in excision repair, whereas the other has a different function required for cell viability. However, we have not eliminated the possibility that RAD3 protein has a single biochemical activity that is involved both in excision repair and in maintenance of cell viability. If different amounts of gene product activity are required for these two aspects of cellular physiology, leaky mutants could retain viability but still demonstrate UV sensitivity.

The 3.2-kb *RAD3* subclone as well as a *RAD3/lac′Z* fusion plasmid, both of which are missing 74 nucleotides at the 3′ end of the coding sequence, conferred normal levels of UV resistance to *rad3* mutants when present on multicopy plasmids. Both of these plasmids also complemented the *RAD3* function essential for viability in haploid cells. Thus, the 3′-terminal region of the gene was not required for either the excision repair of DNA nor for the essential function of *RAD3*. It was surprising that the *lac′Z* fusion near the 3′ end of *RAD3* encoded a functional hybrid protein since the size of this protein was estimated at ~205 kilodaltons, which is more than twice the size of the RAD3 protein. This phenomenon has been reported with other fusion proteins (5, 6, 15); however, we cannot exclude the possibility that some of the hybrid protein was proteolytically cleaved at or near the site of fusion to yield a product that retained normal *RAD3* functions in transformed cells.

In an attempt to gain further insights into structure-function relationships of the *RAD3* gene, we rescued the *rad3-1* and *rad3-2* chromosomal alleles by creating gaps in a centromeric plasmid containing the *RAD3* gene and transforming them into *rad3* mutant strains. We expected that the *rad3-1* and *rad3-2* alleles would be missense and not nonsense mutations since deletions 3′ to the *Eco*RI site located near the 3′ end of the gene (position 2,807 in the sequence; Fig. 3) inactivated both functions of the gene (16), and mapping by rescue with gapped plasmids localized them to regions 5′ of the *Eco*RI site. Each mutation was a single G · C→A · T transition that resulted in a positively charged amino acid. We also established that plasmids carrying the *rad3-1* and *rad3-2* alleles had the same properties as did the chromosomal alleles; i.e., each lost the excision repair function but maintained the viability function. The viability phenotype of the *rad3-1* and *rad3-2* mutants was not due to other mutations or rearrangements in regions of the yeast genome outside the *RAD3* gene; thus, a single point mutation led to the observed phenotype. It is also interesting to note that mutations at codons 236 and 461, separated by a distance of 225 amino acids at the protein level, can each eliminate the repair function while apparently not influencing the viability function. Further detailed mutational analysis of the *RAD3* gene may provide more specific information about the regions required for the excision repair and essential functions.

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