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Molecular Cloning of Eucaryotic Genes Required for Excision Repair of UV-Irradiated DNA: Isolation and Partial Characterization of the *RAD3* Gene of *Saccharomyces cerevisiae*

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We describe the molecular cloning of a 6-kilobase (kb) fragment of yeast chromosomal DNA containing the *RAD3* gene of *Saccharomyces cerevisiae*. When present in the autonomously replicating yeast cloning vector YEp24, this fragment transformed two different UV-sensitive, excision repair-defective *rad3* mutants of *S. cerevisiae* to UV resistance. The same result was obtained with a variety of other plasmids containing a 4.5-kb subclone of the 6-kb fragment. The UV sensitivity of mutants defective in the *RAD1*, *RAD2*, *RAD4*, and *RAD14* loci was not affected by transformation with these plasmids. The 4.5-kb fragment was subcloned into the integrating yeast vector YIp5, and the resultant plasmid was used to transform the *rad3-1* mutant to UV resistance. Both genetic and physical studies showed that this plasmid integrated by homologous recombination into the *rad3* site uniquely. We conclude from these studies that the cloned DNA that transforms the *rad3-1* mutant to UV resistance contains the yeast chromosomal *RAD3* gene. The 4.5-kb fragment was mapped by restriction analysis, and studies on some of the subclones generated from this fragment indicate that the *RAD3* gene is at least 1.5 kb in size.

The biochemistry of the incision of UV-irradiated DNA containing pyrimidine dimers is a key step in excision repair but is only understood in some detail in the procaryotes *Micrococcus luteus* (13–15, 17) and phage T4-infected *Escherichia coli* (9, 13, 23, 25, 28, 35, 43). In uninfected *E. coli*, the products of three unlinked genes (*uvrA*, *uvrB*, and *uvrC*) are required for incision of damaged DNA (36). *E. coli* cells normally contain very small amounts of these gene products, and this quantitative limitation has complicated their extensive purification and detailed characterization. However, recent efforts in a number of laboratories have resulted in the successful cloning and expression of all three genes (27, 31–33, 37, 45). This experimental approach has yielded interesting information about these genes and their products and shows promise of overcoming the quantitative problems encountered in protein fractionation with conventional strains of *E. coli*.

With eucaryotes, progress has been much more limited. Cells from humans suffering from the disease xeroderma pigmentosum show defective repair of UV-irradiated DNA, and a large number of subjects studied fall into one of seven complementation groups defined by cell fusion analyses (see references 10 and 11 for recent reviews). Studies on the ability of cells from each group to effect incision of their DNA after UV irradiation in vivo indicate that at least five of the seven groups are defective (46), suggesting significant complexity in the biochemistry of DNA incision in normal human cells. Such genetic complexity appears to be a feature of other eucaryotic cells that have been less extensively investigated, including Chinese hamster ovary cells (4, 41, 42) and *Drosophila melanogaster* (3, 16).

The yeast *Saccharomyces cerevisiae* is a particularly attractive organism for the study of the molecular mechanism(s) of pyrimidine dimer excision in eucaryotes. In contrast to human cells in culture, yeast cells can be grown quickly and relatively inexpensively in the large quantities required for most biochemical studies. Also, in recent years this organism has been investigated as a model for the regulation of gene expression, and numerous techniques are now available for gene cloning and transformation in yeasts (1). *S. cerevisiae* has also been the subject of extensive genetic analyses, and over 80 loci affecting sensitivity to physical or chemical agents known to promote DNA damage have

TABLE 1. Bacterial and yeast strains

Strain	Relevant genotype[a]	Source
Bacterial		
HB101	*hsdR hsdM*	J. Rubenstein, Stanford University
LE392	*hsdR hsdM*$^+$	J. Rine, Stanford University
SR73	*uvrA6 recA13*	S. Lloyd, Stanford University
SR58	*uvrB5 recA56*	S. Lloyd, Stanford University
SR57	*uvrC34 recA56*	N. Sargentini, Stanford University
Yeast		
SX46A	*MATa ura3-52 trp1-289 his3-832 ade2 RAD*$^+$	J. Rine, Stanford University
XR270-34C	*MATa his4-580*^{a} *trp1*^{a} *tyr1*^{o} *lys2*^{o} *ade2*^{o} *RAD*$^+$	J. Rine, Stanford University
S288C	*MATα gal2 RAD*$^+$	R. Reynolds, Harvard University
LN1	*MATα rad1-19 ura3-52*	This study
LN2	*MATα rad2-2 ura3-52 trp1-289*	This study
LN3	*MATα rad3-1 ura3-52 his3-832 ade2*	This study
LN4	*MATα rad4-3 ura3-52 trp1-289*	This study
LN14	*MATα rad14-1 ura3-52 trp1-289 his3-832*	This study

[a] The superscripts "a" and "o" indicate amber and ochre mutations, respectively.

been identified (18). Many of these fall into three major independent (so-called epistasis) groups, of which one (the *RAD3* group) contains a number of loci required for the normal excision repair of pyrimidine dimers (18).

In previous studies from this laboratory it has been shown that strains carrying mutations in the *RAD1*, *RAD2*, *RAD3*, and *RAD4* genes (all members of the *RAD3* epistasis group) do not show detectable incision of UV-irradiated DNA in vivo (29, 30). These results were confirmed by Wilcox and Prakash (44), who also showed that the *RAD10* gene is required for incision of UV-irradiated DNA in vivo. Collectively these data suggest that, as it is in human cells, the biochemistry of the incision of damaged DNA in yeasts is complex. Numerous attempts to demonstrate a UV DNA incising activity in cell-free preparations of *S. cerevisiae* have been unsuccessful in our hands (R. J. Reynolds, J. D. Love, and E. C. Friedberg, unpublished data). We have therefore molecularly cloned some of the *RAD3* group genes to isolate and characterize their products and reconstitute a catalytically active UV DNA incising activity in vitro. We report here the molecular cloning and preliminary characterization of the *RAD3* gene from *S. cerevisiae*.

MATERIALS AND METHODS

Yeast and bacterial strains and plasmids. The yeast and bacterial strains used in this study are shown in Table 1. *S. cerevisiae* S288C (*RAD*$^+$), *rad1-19*, *rad2-2*, *rad3-1*, *rad4-3*, and *rad14-1* were provided by R. Reynolds, Department of Radiobiology, Harvard University. These *rad* mutants were originally isolated from strain S288C and have been extensively backcrossed to maximize their isogenicity with the wild-type strain (38). The *rad3-2* mutant was obtained from the Yeast Genetic Stock Center, Berkeley, Calif. Strains for transformation with plasmids were constructed by mating *rad* mutants to strain SX46A (*ura3-52 trp1-289 ade2-1 his3-832*) (kindly provided by J. Rine, Department of Biochemistry, Stanford University) and isolating appropriate haploid strains by standard procedures (24).

E. coli LE392 (*hsdR hsdM*) (provided by J. Rine) and *E. coli* HB101 (*hsdR hsdM*) (provided by J. Rubenstein, Department of Biochemistry, Stanford University) were used for the propagation of plasmids. *E. coli* strains carrying mutations *uvrA6 recA56*, *uvrB5 recA56*, and *uvrC34 recA56* were obtained from N. Sargentini, Department of Radiology, and S. Lloyd, Department of Biological Sciences, Stanford University. Plasmids YRp16, YRp17, and YIp5 were obtained through the courtesy of R. W. Davis, Department of Biochemistry, Stanford University.

Culture media. Yeast strains were grown selectively in medium containing 0.17% (wt/vol) yeast nitrogen base and 2% (wt/vol) dextrose, supplemented with the required nutrients except uracil (minimal medium). For nonselective growth, YPD medium (1% [wt/vol] yeast extract [Difco], 2% [wt/vol] glucose, and 2% peptone [Difco]) was used. Agar was added to 2% (final concentration) for growing the cultures on plates. Minimal medium agar was used for measurement of cell viability after UV irradiation. For selection of plasmid-containing transformants, *E. coli* strains were grown in L broth supplemented with ampicillin (50 μg/ml) or tetracycline (15 μg/ml).

Preparation of DNA. *E. coli* HB101, carrying DNA fragments representative of the entire yeast genome as inserts in the yeast cloning vector YEp24 (5), was obtained through the courtesy of Marion Carlson, Department of Human Genetics and Development, Columbia University.

Rapid lysates of yeast cultures containing self-replicating or integrated plasmid DNA were prepared by suspending 10-ml cultures of cells in 0.2 ml of 50 mM Tris-hydrochloride (pH 7.5)–50 mM EDTA–1% sodium dodecyl sulfate. Siliconized glass beads were added up to the meniscus, and the suspensions were mixed in a Vortex stirrer for 60 s. The resulting lysates were brought to a volume of 0.5 ml with water and extracted three times with buffered phenol (pH 8.0) and twice with anhydrous ether. The DNA was precipitated by the addition of 1/10 volume of 3.0 M sodium acetate (pH 5.5) and 2 volumes of ethanol. The precipitate was dissolved in 0.01 M Tris-hydrochloride (pH

7.5)–1.0 mM EDTA and 10 μg of DNase-free RNase per ml and used in this form to transform *E. coli* and for hybridization experiments.

Rapid lysates of *E. coli* containing plasmids were prepared by suspending the cells in 15% sucrose–50 mM EDTA–50 mM Tris-hydrochloride (pH 8.0) and lysozyme (1.0 mg/ml) and incubating them at 23°C for 30 min. A 0.3-ml amount of 50 mM Tris-hydrochloride (pH 8.0)–50 mM EDTA–0.1% Triton X-100 was added to the suspension, and the cells were heated to 55°C for 15 min. After lysis had occurred, the preparation was centrifuged for 60 min at 15,000 rpm in an SS-34 Beckman rotor containing adaptors for microfuge tubes, extracted with phenol and then with ether, and precipitated by the addition of 1/10 volume of 3.0 M sodium acetate (pH 5.5) and 2 volumes of ethanol. The ethanol precipitates were dissolved as described above.

Transformation of cells with DNA. Transformation of *E. coli* with plasmid DNA was carried out by the method of Cohen et al. (7) as described by Okayama and Berg (26). For transformation of yeast cells the procedure of Hinnen et al. (20) was followed, except that cells were converted to spheroplasts by treatment with 2% glusulase for 1 to 2 h. After the addition of DNA and polyethylene glycol, cells were incubated for 5 min at 23°C, then centrifuged at 3,000 rpm in a GLC 1 table-top centrifuge for 3 min, and suspended in 1.0 ml of 1.0 M sorbitol. Regeneration agar (0.17% yeast nitrogen base without amino acids, 2% dextrose, 1.0 M sorbitol, 3% agar, supplemented with histidine [20 μg/ml], tryptophan [30 μg/ml], and adenine (30 μg/ml]) was added to the cells, mixed gently, and poured onto minimal medium plates. Transformants were generally visible after 3 days of incubation at 30°C and could usually be picked by 5 days after plating, depending on the particular plasmid and strains used.

Characterization of DNA. Cesium chloride-ethidium bromide gradient centrifugation of plasmid DNA, restriction enzyme digestion, gel electrophoresis, ^{32}P labeling of DNA by nick translation, and DNA-DNA hybridization were performed as described by Davis et al. (8). Restriction enzymes were purchased from Bethesda Research Laboratories, Bethesda, Md., or from New England Biolabs, Boston, Mass.

RESULTS

Strategy for the isolation of UV-resistant yeast colonies. The molecular cloning of *RAD* genes from *S. cerevisiae* was accomplished by screening for colonies of UV-sensitive *rad* strains that showed the phenotype of enhanced resistance to UV radiation after being transformed with plasmids containing putative yeast chromosomal DNA inserts representative of the entire yeast genome. A priori such an identification method seemed to be feasible, since at a UV dose of 15 J/m^2 the survival of the wild-type and most mutant strains of interest to us (*rad1-19*, *rad2-2*, *rad3-1*, *rad4-3*, and *rad10-1*) differs by 4 to 5 orders of magnitude (29, 30).

The gene pool used was constructed by Carlson and Botstein (5) by ligating a partial *Sau*3AI digest of yeast chromosomal DNA into the *Bam*HI site of the cloning vector YEp24 (2). This vector contains pBR322 DNA, including the Tc^r and Ap^r genes, the yeast chromosomal *URA3* gene, and a region of yeast endogenous plasmid DNA required for autonomous replication in yeast. Approximately 21,000 *E. coli* transformants were isolated, collectively constituting a complete yeast gene pool.

Haploid strains were constructed by appropriate crosses to contain the *ura3-52* and *rad3-1* alleles as well as the other genetic markers indicated in Table 1. Yeast cultures were transformed with plasmid YEp24 containing yeast chromosomal DNA inserts, plated on minimal medium plates, and incubated at 30°C. Colonies that grew under these selective conditions presumably contained plasmid DNA which had expressed the *URA3* gene. Small samples of individual colonies were transferred as a thin film of cells to minimal medium master plates by using sterile toothpicks and replica plated onto agar plates containing complete medium since, after UV irradiation, cells grew faster on the latter medium. The replica plates were exposed to a dose of UV radiation (12.5 J/m^2) determined to be lethal to a strain carrying the *rad3-1* mutation but not to a strain with a UV-resistant phenotype.

A *rad3-1 ura3 his3 ade2* haploid strain of *S. cerevisiae* was transformed with the *Sau*3AI gene pool. After screening ~2,000 colonies, about 40 colonies that showed partial UV resistance were identified. All of these were retested by isolating samples of each colony from the minimal medium master plate and suspending each in sterile water. Each culture was streaked onto an agar plate containing complete medium as a single line across the entire plate; *RAD3* and *rad3* control cultures were streaked onto each plate as well. Different sectors of the plates were exposed to graded doses of UV radiation between 0 and 50 J/m^2, and the plates were incubated at 30°C overnight. By this test a single colony still showed considerable UV resistance relative to the *rad3-1* control but was qualitatively somewhat UV sensitive relative to *RAD3* (Fig. 1).

The partial resistance to UV radiation (Fig. 1) reflects a mixed population of transformants, only some of which carried the UV resistance determinant (data not shown). Cotransformation in yeasts is a frequent event (19, 22, 39), and subculturing of this colony under selective growth conditions was necessary to isolate one with normal UV resistance that contained only a single plasmid carrying both the *URA3* and UV resistance determinants. Subcultured colonies on minimal medium plates were streaked onto YPD agar plates, and samples of individual colonies were transferred in an ordered pattern

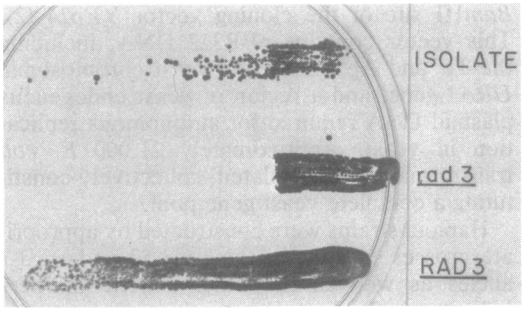


FIG. 1. Retesting of the originally isolated UV-resistant colony by a qualitative assay. A small sample of the UV-resistant colony was taken from a master plate and suspended in 0.5 ml of sterile water. Colonies of control *rad3* and *RAD3* strains were resuspended in separate tubes of water. A sample of each suspension was streaked across the surface of a YPD plate and allowed to dry. Selected parallel regions of the plate were exposed to graded doses of UV radiation between 0 and 50 J/m^2 (from right to left).

to a minimal medium plate and replicated onto two separate YPD plates, one of which was exposed to UV radiation. The plasmid YEp24 replicates autonomously and segregates randomly to daughter cells (2). Thus, if the acquired resistance of cells to UV radiation was due to expression of a gene on a single plasmid, mitotic loss of the plasmid should result in a strict correlation between loss of UV resistance and loss of the ability to grow on medium without uracil. A colony demonstrating this cosegregation of plasmid markers was identified, and isolation of plasmid DNA from this colony and its propogation in *E. coli* demonstrated the presence of a single plasmid, which was designated pNF3000.

When plasmid pNF3000 was introduced into the UV-sensitive mutant *rad3-1*, the transformants demonstrated a normal resistance to UV radiation as qualitatively measured by the streak test described above. Quantitative UV survival data for *rad3-1* cells transformed with either YEp24 or pNF3000 show complementation of UV resistance to normal levels by the latter plasmid (Fig. 2). This plasmid also transformed a different *rad3* mutant allele (*rad3-2*) to normal levels of UV resistance (data not shown). No enhanced UV resistance was observed after transformation of *rad1-19*, *rad2-2*, *rad4-3*, or *rad14-1* strains, all of which contain the *ura3-52* mutation (data not shown). In addition, all of the *rad3-1* colonies transformed with purified pNF3000 DNA showed a strict correlation between the loss of UV resistance and the requirement for uracil for growth.

Restriction mapping and subcloning of pNF3000. Plasmid pNF3000 DNA was digested separately with the restriction enzymes *Eco*RI, *Bgl*II, *Sst*I, and *Bam*HI, and the products were subjected to agarose gel electrophoresis. A comparison of the additive molecular weights of the fragments generated in each case with those generated from the original cloning vector showed that pNF3000 contains an insert of about 6 kilobases (kb). A restriction enzyme analysis of the plasmid with *Eco*RI enzyme generated 6 fragments, labeled A–F in order of decreasing size. The correct orientation of these DNA fragments in the plasmid was determined by subcloning partial *Eco*RI digests of pNF3000

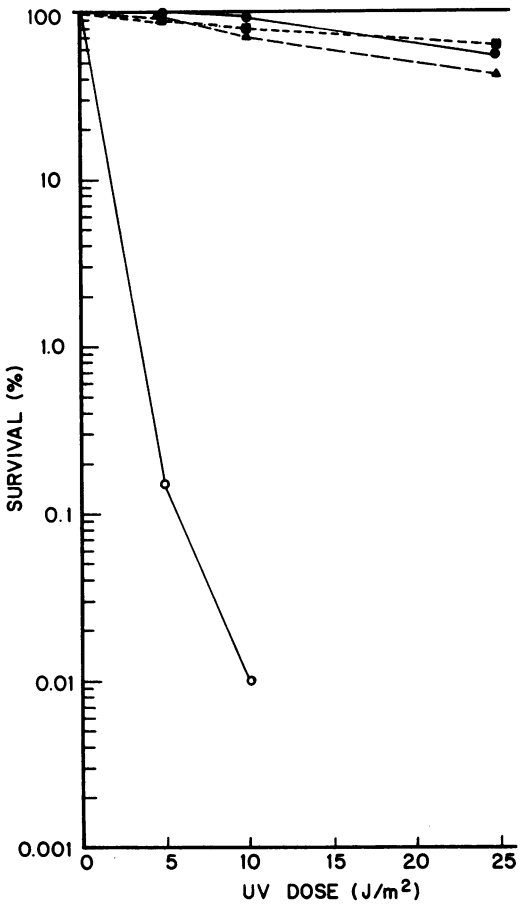


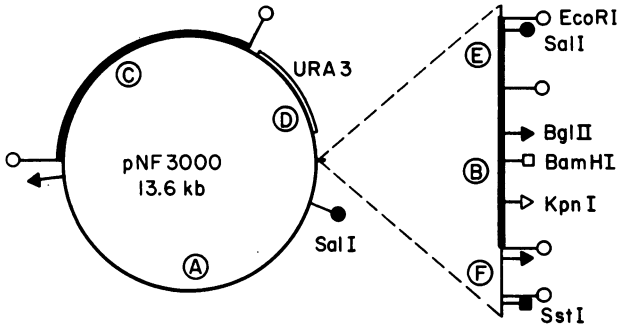
FIG. 2. UV survival of yeast strains transformed with various plasmids. Yeast cultures transformed with the relevant plasmids were grown overnight in minimal medium to a density of 2×10^7 to 4×10^7 cells per ml. Appropriate dilutions were then spread onto agar plates containing minimal medium and exposed to UV radiation at the doses indicated. Colonies were counted after 4 days of incubation at 30°C. ■, Strain SX46A (*RAD3*) transformed with YEp24; ●, strain *rad3-1* transformed with pNF3000; ▲, strain *rad3-1* transformed with pNF3207 (integrating plasmid; see the text); ○, strain *rad3-1* transformed with YEp24.

into the *Eco*RI site of the plasmid YRp16. DNA prepared from subclones was digested to completion with *Eco*RI enzyme and analyzed by gel electrophoresis to yield the unique orientation of fragments A–F (Fig. 3).

Some of the subclones were tested for their ability to confer UV resistance to strain *rad3-1*. A positive result was obtained with two of the subclones, both of which contained only fragments B and E (3.15 and 1.35 kb, respectively) in common (Fig. 3). Subclones containing only fragment B or E did not confer UV resistance. Thus, the gene that conferred UV resistance to *rad3-1* is presumably spanned by these two fragments and inactivated by cleavage at the central *Eco*RI site (Fig. 3). The 4.5-kb subfragment containing fragments B and E was subjected to further restriction mapping, and the sites identified are shown in Fig. 3.

The 4.5-kb DNA subfragment contains the *RAD3* gene. Experiments were performed to demonstrate more definitively that the 4.5-kb DNA subfragment described above contains the *RAD3* gene rather than some other function that confers UV resistance to *rad3* strains. Such a result could arise, for example, if the *rad3-1* strain contained a suppressible mutation and we had cloned a suppressor gene. The experimental approach that we used is based on the observation by others (19, 20) that yeast plasmids that cannot replicate autonomously integrate into the yeast genome by site-specific recombination with homologous DNA sequences. The plasmid can then be physically and genetically mapped in the genome to establish the identity of the cloned gene in question.

The 4.5-kb fragment was subcloned into the vector YIp5 to yield plasmid pNF3207, which is 10 kb in size (Fig. 4A). This plasmid lacks a sequence required for autonomous replication and hence can transform yeast cells only by integrating into the genome (34). A *rad3-1* mutant (strain LN3, Table 1) was transformed with plasmid DNA, transformants were identified as URA^+, and stable RAD^+ (see Fig. 2 for UV survival curve) URA^+ integrants were selected. DNA was extracted from cultures of these integrants and from cultures of *RAD3* and *rad3* nonintegrant control strains and restricted with the enzyme *Sst*I. The restricted DNA was hybrid-



Plasmid	EcoRI fragments subcloned	Transformation to UV resistance
pNF 3002	D E	-
pNF 3005	D E B	+
pNF 3010	A C	-
pNF 3031	E B	+
pNF 3032	B F	-
pNF 3033	F A	-
pNF 3039	B F A	-
pNF 3014	E	-
pNF 3030	B	-
	A C D E B F A	

FIG. 3. Subcloning and restriction mapping of the plasmid pNF3000. Plasmid DNA was cleaved with *Eco*RI to yield partial digests which were subcloned into the yeast vector YRp16. Transformants were selected as Tc^r colonies. A set of subclones containing overlapping partial digests was identified and used to transform a *rad3-1 ura3-52* strain. URA^+ transformants were selected and tested for their resistance to UV radiation. The *Eco*RI restriction map (fragments A–F) was deduced from this analysis, the results of which are shown in the lower half of the figure. Sites for other restriction enzymes were mapped by multiple digests of pNF3000 or various subclones in YRp16. The bold line in the insert represents the 4.5-kb subfragment defined by fragments B and E.

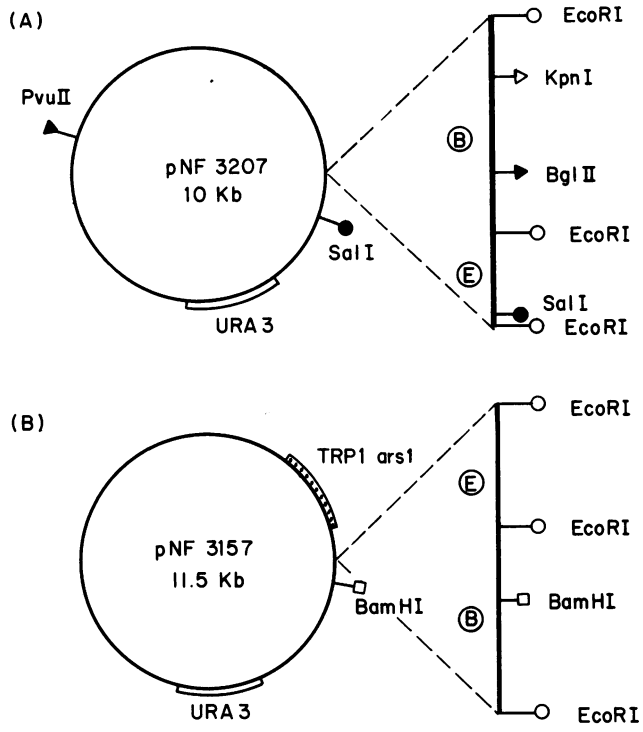


FIG. 4. Structure and construction of plasmids pNF3207 (A) and pNF3157 (B). pNF3207 was constructed by cloning partial *Eco*RI digests of pNF3000 into the *Eco*RI site of YIp5. This plasmid expresses genetic functions after integration into the yeast chromosome and was used in some of the integration experiments described in the text. pNF3157 was constructed by cloning partial *Eco*RI digests of pNF3000 into the *Eco*RI site of YRp17. This plasmid was used to effect the deletion of the 1.7-kb segment of DNA between the two *Bam*HI sites shown.

ized against a ^{32}P-labeled plasmid probe (pBR322) containing the 4.5-kb subfragment by the Southern blot technique.

Plasmid pNF3207 cannot integrate at the chromosomal *ura3* site because the *ura3-52* mutation used in these studies has a significant alteration that precludes homologous recombination at this site (34). Hence, if the plasmid integrated by homologous recombination at the site of the cloned 4.5-kb fragment within the genome (putatively the *rad3* site), we would expect to observe hybridization of the probe to a unique fragment of DNA containing the integrated plasmid and its homolog in tandem array, provided the restriction enzyme did not cut within either sequence. Plasmid pNF3207 does not contain an *Sst*I-sensitive site; thus, the restriction fragment identified by hybridization should be ~10 kb larger (the size of the integrated plasmid) than the unique restriction fragment generated by digestion of the *rad3* and *RAD3* control DNA (lanes 2 and 3, Fig. 5A) to which the probe should also hybridize. The results expected were obtained and are shown in lane 1 of Fig. 5A for one of the integrants.

Further corroboration of this result was obtained by carrying out the identical experiment with the restriction enzyme *Pvu*II. We established that the integrating plasmid pNF3207 has a single site for this enzyme in the pBR322 region of the vector (Fig. 4A). Thus, whereas we again expected to observe hybridization to a single restriction fragment from control DNA (*RAD3* or *rad3*), in this case hybridization to two unique restriction fragments of integrant DNA should be observed. This result was obtained for eight integrants isolated, and the sizes of these restriction fragments (12 and 9.5 kb) gave a cumulative value of 21.5 kb (Fig. 5B). This value is ~9.5 kb larger than the size of the fragments from the control DNA (12.5 kb) identified by the hybridization probe and close to that of the integrating plasmid.

To determine whether pNF3207 integrated at or very close to the *rad3* gene, we mated two UV-resistant integrants to an *RAD3* strain. Diploids were selected by growth on appropriate selective plates and allowed to sporulate. Asci were isolated, and the spores from each ascus were subjected to tetrad analysis to determine the segregation of UV resistance and the requirement for tryptophan, an unlinked marker.

If plasmid pNF3207 had integrated near the *rad3* site, there should be a very tight genetic linkage between the *rad3* (host) and *RAD3* (cloned) genes. Thus, essentially all of the spores isolated from a cross of such integrants with a *RAD3* strain should be phenotypically UV resistant. All of 21 complete tetrads tested showed this result (Table 2). In addition, the *TRP* and *trp* genes segregated $2^+:2^-$ in these crosses (Table 2), indicating that meiotic segregation was normal in all asci tested. In control experiments the untransformed *rad3-1* strain was crossed with an *RAD3* strain. As expected, *RAD3*, *TRP1*, and *URA3* genes segregated $2^+:2^-$ (Table 2).

Further subcloning of the 4.5-kb subfragment. The 4.5-kb fragment was subcloned into the vector YRp17 (Fig. 4B). The resulting plasmid (pNF3157) was restricted at the *Bam*HI sites and self-ligated at low DNA concentrations, resulting in deletion of the 1.7-kb region of DNA between the two *Bam*HI sites (Fig. 4B). Plasmids with this deletion were propogated in *E. coli* HB101 cells and tested for their ability to transform the *rad3-1* strain to UV resistance. No UV-resistant colonies were observed, suggesting that the *Bam*HI site in the 4.5-kb fragment resides within the *RAD3* gene. This gene therefore extends at least from the *Eco*RI site at the junction between fragments B and E to the *Bam*HI site in fragment B (Fig. 3 and 4B), a distance of 1.5 kb.

Complementation of *E. coli uvr*⁻ strains. Strains of *E. coli* carrying the *uvrA*, *uvrB*, or *uvrC* mutation (Table 1) were transformed with plasmid pNF3000. None of the strains showed recovery of UV resistance as compared with controls. However, it was not specifically determined whether yeast genes on the plasmid were expressed in the *E. coli* strains.

DISCUSSION

The protocol described here is clearly useful for the identification of plasmids containing DNA inserts that confer enhanced UV resistance to *rad3-1* mutants of *S. cerevisiae*. This screening procedure is somewhat laborious, since it involves the transferral of individual colonies to master plates and replica plating to a second set of plates for irradiation. However, mass screening protocols that involve selection of transformants for UV resistance were not successful in this study.

Plasmid pNF3000 contains a 6-kb yeast DNA insert. Partial *Eco*RI fragments of the plasmid were subcloned and used to construct a restriction map. Only subclones containing two *Eco*RI fragments, called B and E (4.5 kb), conferred UV resistance to *rad3* mutants. Smaller subclones generated by *Bam*HI digestion failed to produce this result. We therefore conclude that the cloned *RAD3* gene spans at least the distance between the central *Eco*RI and the *Bam*HI sites (1.5 kb) (Fig. 3 and 4B). Thus, if both the *Bam*HI and *Eco*RI enzymes cleaved within the structural gene, the protein product of the *RAD3* gene has a molecular weight of at least 50,000. Current studies are aimed at defining more precisely the region(s) of the cloned fragment that is essential for the expression and regulation of the *RAD3* gene.

Our experiments indicate that the transformation of *rad3-1* mutants by plasmids containing the cloned DNA is caused by expression of the *RAD3* gene specifically rather than being the result of suppression of the *rad3-1* mutation by a cloned suppressor gene. This was shown by integrating the 4.5-kb subfragment into the genome of a *rad3-1* mutant at its homologous sequence with the integrating vector YIp5. Mating UV-resistant *rad3* integrants to *RAD3* cells and performing tetrad analysis on the resulting spores showed that the two genes were very closely linked. This indicates that the 4.5-kb subfragment underwent integration by homologous recombination at or very near the *rad3* site. Control mating experiments showed normal meiotic segregation of all markers tested.

In addition to these genetic experiments we carried out Southern blot analysis of restricted wild-type yeast DNA and of restricted DNA containing the integrated 4.5-kb fragment. These analyses confirmed that the cloned DNA was of yeast origin and that it had integrated at the expected site of homology, i.e., the *rad3* site. The observation that the size of the restriction fragments from *rad3* and *RAD3* DNA identified

TABLE 2. Genetic analysis of integrant strains

Genetic cross	Selection	Marker segregation (ratio)				
		$4^+:0^-$	$3^+:1^-$	$2^+:2^-$	$1^+:3^-$	$0^+:4^-$
Int1 (*MAT*α) × XR270-34C (*MAT***a**)	*RAD*⁺	12	0	0	0	0
	TRP⁺	0	0	12	0	0
Int2 (*MAT*α) × XR270-34C	*RAD*⁺	9	0	0	0	0
	TRP⁺	0	0	9	0	0
rad3-1 (*MAT*α) × XR270-34C	*RAD*⁺	0	1	10	0	0
	TRP⁺	0	0	11	0	0
	URA⁺	0	0	11	0	0

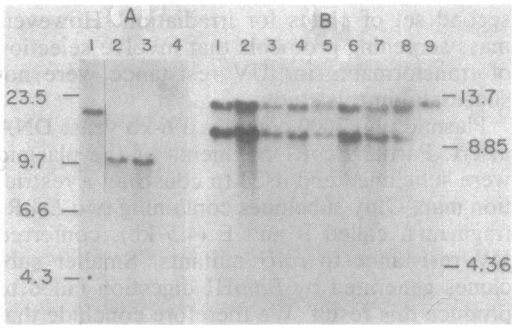


FIG. 5. Southern hybridization analysis of integrant and control strains of yeast. (A) Total genomic DNAs from one strain containing integrated DNA (see the text) and from *rad3* and *RAD3* non-integrant control strains was extracted as described in the text. The DNAs were restricted with the enzyme *Sst*I and electrophoresed on a 0.5% agarose gel. Southern blotting and hybridization against ^{32}P-labeled pBR322 DNA containing the 4.5-kb cloned subfragment were carried out as described by Davis et al. (8). Lanes: 1, integrant DNA; 2, *RAD3* DNA; 3, *rad3* DNA; 4, unrestricted integrant DNA. The molecular weights ($\times 10^{-3}$) of marker fragments obtained by *Hin*dIII digestion of λ DNA are shown at the left. The band in lane 1 is separated from those in lanes 2 and 3 by a distance equivalent to ~9 kb. (B) The same analysis was performed with eight integrant strains and one *RAD3* control strain, except that the enzyme *Pvu*II was used and the restricted DNA was electrophoresed through a lower-percentage gel. Lanes: 1–8, integrant DNA; 9, *RAD3* DNA. The molecular weights ($\times 10^{-3}$) of previously characterized DNA fragments are shown at the right. The single band in lane 9 is 12.5 kb in size. The upper and lower bands in lanes 1–8 have sizes of 12.0 and 9.5 kb, respectively.

by hybridization were indistinguishable also suggests that the *rad3-1* mutant does not contain any gross alteration in DNA structure at the *rad3* locus that is detectable by this technique. Since no other hybridization bands were detected, we further conclude that the *RAD3* gene does not share extensive sequence homology with other sites in the yeast genome.

The possibility that we cloned a tRNA suppressor located very close to the *RAD3* gene in the yeast chromosome was considered highly unlikely for the following reasons: (i) strains carrying pNF3000 grow normally as compared with wild-type strains; other strains carrying some tRNA suppressors generally do not (12); (ii) the minimum size of the gene conferring UV resistance to *rad3* mutants is 1.5 kb, far larger than the small tRNA suppressor genes described; (iii) no other genetic markers used in this study were affected by the presence of the cloned DNA, including other *RAD* mutations, amber mutations in the *HIS* and *TRP* genes, and ochre mutations in the *TYR* and *ADE* genes (unpublished data); (iv) two independent *RAD3* mutant alleles were complemented with equal efficiency; and (v) the *rad3-1* mutant used in our study was originally isolated from strain S288C (38), the same strain used for the construction of the *Sau*3AI gene pool. If S288C contains a suppressor of *rad3-1*, it should not have been possible to isolate the *rad3-1* mutant.

Our results show that when the *rad3-1* strain is transformed with either integrating or autonomously replicating plasmids containing the cloned yeast DNA, normal levels of UV resistance are observed. The latter group of plasmids normally exist at a high copy number (1, 40). Assuming that this situation results in overexpression of the *RAD3* gene, as is true of other genes that have been studied (6, 21), we conclude that such putative over-expression is not lethal to the host or else that the expression of the cloned gene is regulated in *S. cerevisiae*.

Using the same screening protocol, we also cloned a DNA fragment that complements *rad2* mutants of *S. cerevisiae*, a DNA fragment that complements the *rad1-1* mutation (E. Yang, L. Naumovski, and E. C. Friedberg, manuscript in preparation), and one that complements the *rad4-3* mutation (G. Pure and E. C. Friedberg, unpublished data). The characterization of these will be described elsewhere. The cloning and expression of other *RAD* genes is in progress. Studies on the expression of these genes may provide information on the gene products required for the incision of UV-irradiated DNA in *S. cerevisiae* specifically and perhaps in eucaryotes in general.

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