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Genetics

A yeast excision-repair gene is inducible by DNA damaging agents

(*RAD2* gene/gene fusions/mRNA/β-galactosidase/*Saccharomyces cerevisiae*)

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ABSTRACT **Plasmids containing various *RAD-lacZ* gene fusions were integrated into the chromosome of haploid yeast cells. These integrant strains were tested for expression of *Escherichia coli* β-galactosidase after treatment with agents that damage DNA or interfere with normal DNA replication. We did not observe induction of single-copy *RAD1-lacZ* or *RAD3-lacZ* fusion genes under the experimental conditions used. However, exposure of cells containing an integrated *RAD2-lacZ* fusion gene to UV-radiation, γ-radiation, 4-nitroquinoline 1-oxide, or nalidixic acid resulted in 4- to 6-fold enhanced expression of β-galactosidase. Induction of the *RAD2* gene after treatment of untransformed cells with 4-nitroquinoline 1-oxide was confirmed by direct examination of *RAD2* mRNA. Lower levels of induction (≈50%) were observed after treatment of cells with other chemicals. Induction of the *RAD2-lacZ* fusion gene was also observed in cells transformed with single-copy and multicopy autonomously replicating plasmids.**

Mutations at a number of loci in the yeast *Saccharomyces cerevisiae* cause increased sensitivity to killing by UV-radiation and reduced ability to excise pyrimidine dimers from DNA (see ref. 1). Defects in at least five of these genes (*RAD1, RAD2, RAD3, RAD4*, and *RAD10*) result in the inability to carry out specific incision of DNA, an early event in nucleotide excision repair (2, 3). These results suggest that the products of these five *RAD* genes are required for incision and/or preincision events associated with excision repair of base damage from chromatin in yeast cells. A requirement for multiple proteins for the incision of damaged DNA also has been demonstrated in the prokaryote *Escherichia coli* (see ref. 4). There is good evidence that the genes encoding some of these proteins (*uvrA, uvrB*, and *urvD*) are part of the inducible SOS regulon that responds to DNA damage (see ref. 4). The inducibility of the *uvrC* gene is controversial (5), and there is no evidence for enhanced expression of *polA* after exposure of cells to DNA damage.

The *RAD1, RAD2, RAD3*, and *RAD10* genes of *S. cerevisiae* have been isolated in this laboratory (6–9) and by others (10–14) by using molecular cloning techniques. In the present study we investigated the possible induction of some of these genes after DNA damage, using *RAD-lacZ* fusions that express *E. coli* β-galactosidase in yeast. After the integration of a *RAD2-lacZ* fusion gene into a Rad$^+$ yeast strain, we observed a significant increase in the level of β-galactosidase in cells exposed to UV-radiation, γ-radiation, 4-nitroquinoline 1-oxide (4NQO), or nalidixic acid. Using 4NQO treatment as an example, we show that this response is strictly dependent on active protein synthesis and that increased levels of *RAD2* transcripts are observed after treatment of untransformed cells. Lower levels of induction were observed in cells treated with other agents that damage DNA or interfere with normal DNA replication. Induction of integrated single-copy *RAD1-lacZ* or *RAD3-lacZ* genes was not observed under these experimental conditions, confirming the results of previous studies (15, 16).

MATERIALS AND METHODS

Yeast and Bacterial Strains and Plasmids. The haploid strain SX46a (*MATa ura3-52 trp1-289 his3-832 ade2*) is maintained in our laboratory stocks. *E. coli* strain HB101 was used for propagation of plasmids. Plasmid pSR16 containing the *DIN1-lacZ* fusion was a gift from S. Ruby and J. Szostak. Plasmids YEp24, YIp5, YCp5, and pMC874 were obtained from R. W. Davis.

Culture Media and Chemicals. Yeast and bacteria were grown as described (6). 4NQO (Sigma) was prepared and stored as a stock solution at 0.5 mg/ml as described by Ruby and Szostak (17). Mitomycin C and bleomycin (Bristol Laboratories, Syracuse, NY) and nalidixic acid (Aldrich) were added to final concentrations as dry reagents. Methyl methanesulfonate was from Aldrich, and methotrexate was from Sigma. Nitrogen mustard (mechlorethamine) was from Merck and was prepared as a 20 mM stock solution immediately prior to use.

Preparation of DNA and Transformation of Cells. Transformation of cells and preparation of lysates were as described (6, 18, 19). CsCl-banded plasmid DNA was used for all integrative yeast transformations and was linearized to target integration to specific locations as described (20). Copy number and localization of integrated fusion genes were established by DNA·DNA hybridization.

Characterization of DNA. Restriction enzymes were purchased from Bethesda Research Laboratories. S1 nuclease was from Sigma, T4 DNA polymerase and the Klenow fragment of DNA polymerase I were from Bethesda Research Laboratories, and T4 DNA ligase and *Bgl* II linkers were from New England Biolabs. Gel electrophoresis of DNA and DNA·DNA hybridization were performed as described by Davis *et al.* (21). ^{32}P labeling of DNA was carried out with mixed random primers to prime DNA synthesis by the method of Feinberg and Vogelstein (22). DNA sequencing was by the Maxam–Gilbert procedure (23).

Construction of *lacZ* Reading Frame Plasmids. The construction of plasmids containing a portion of the *E. coli lacZ* gene (24) for fusion with different *RAD* genes is shown diagrammatically in Fig. 1. Kanamycin-resistant plasmids were screened for those that lost the *Bam*HI site and acquired a unique *Bgl* II site. Nucleotide sequence analysis confirmed that plasmids from the three groups representative of each DNA polymerization reaction differed one from the other by a single nucleotide. A single plasmid from each group was designated pRF1, pRF2, or pRF3, respectively. In order to expand the versatility of these reading frame plasmids, they were tailored into the vector pUC18 to place a restriction site polylinker 5′ to the modified *lacZ* gene.

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Abbreviation: 4NQO, 4-nitroquinoline 1-oxide.

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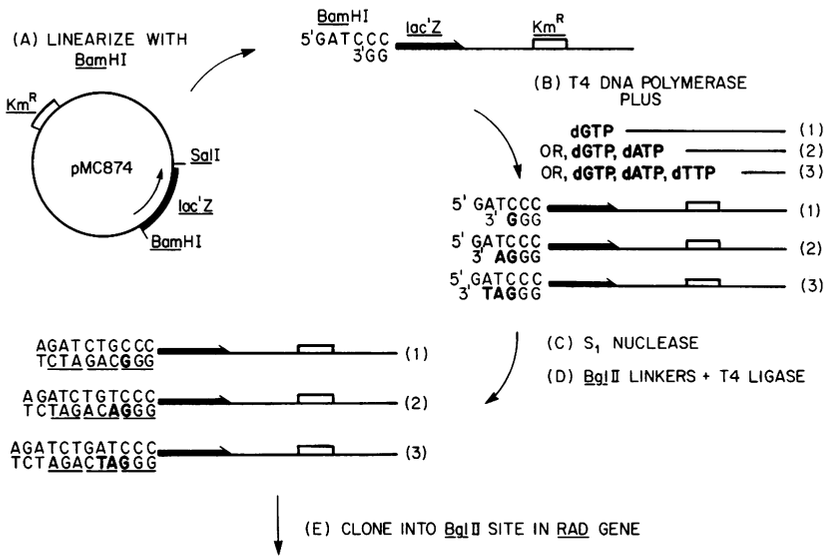

FIG. 1. Construction of *lacZ* reading frame plasmids pRF1, pRF2, and pRF3. Plasmid pMC874, containing the *E. coli lacZ* gene (▬) deleted of its promoter and the first seven codons of the gene, was treated in sequential steps as outlined. The three plasmids obtained possessed a unique *Bgl* II site that was 1, 2, or 3 base pairs upstream of the *"CCC"* codon of the *lacZ* gene.

Construction of *RAD-lacZ* Fusion Genes. *RAD-lacZ* fusions were generated by cloning entire reading frame plasmids into the coding regions of various *RAD* genes. For each *RAD* gene a set of six fusion plasmids was generated, representing insertions of the *lacZ* gene in either orientation and in all three translational reading frames. Productive fusion plasmids were designated as pRF1001 (*RAD1-lacZ*), pRF2001 (*RAD2-lacZ*), and pRF3001 (*RAD3-lacZ*) and were shown to be the constructs predicted from the established nucleotide sequence of the individual genes (7, 15, 25). A second *RAD2-lacZ* fusion (pRF2002) utilized an *Eco*RV site in the *RAD2* coding region and expressed more β-galactosidase activity than did pRF2001. This fusion was used for all experiments described here. Integrating (pRFI1003, pRFI2003, and pRFI3003) and centromeric (pRFC1004, pRFC2004, and pRFC3004) fusion plasmids were generated by subcloning into *Sal* I sites on the vectors YIp5 and YCp19, respectively.

β-Galactosidase Assays. A qualitative filter assay for β-galactosidase (15) was used to screen for plasmids containing the *E. coli lacZ* gene in the correct transcriptional and translational alignments relative to the *RAD* genes. Quantitative assays for β-galactosidase in liquid cultures of yeast were carried out with extracts prepared as described (17). Enzyme activity was measured by using *O*-nitrophenyl β-D-galactopyranoside or 4-methylumbelliferyl β-D-galactoside as substrates. The product of reaction with the latter substrate is fluorescent (26) and was measured with an Eppendorf photofluorometer standardized with a solution (1 μg/ml) of 4-methyllumbelliferone. β-Galactosidase activity (based on either assay) is expressed as specific activity (units per cell density equivalent).

Messenger RNA Analysis. Individual cultures (500 ml) of untransformed SX46*a* cells or SX46*a* cells transformed with plasmid pSR16 containing the *DIN1-lacZ* fusion gene (to serve as a positive control for induction) were grown in minimal medium to OD_{600} = 0.8–1.0. 4NQO was added to a final concentration of 0.25 μg/ml to some of the cultures, which were then incubated for 30 or 60 min. Yeast RNA and *RAD2* DNA probe were prepared and used in S1 nuclease reactions as described (25) except that hybridizations were carried out at 46°C in 20 μl of 90% recrystallized formamide for 15 hr, and S1 nuclease digestion was at pH 6.0. The proportion of poly(A)$^+$ RNA in preparations of total RNA was calculated by hybridization with [^{3}H]poly(U) as described by Bishop *et al.* (27). Densitometry measurements were performed on a Helena Laboratories Quick Scan R & D densitometer.

Measurement of Induction After DNA Damage. Yeast cells transformed with integrated *RAD-lacZ* fusions were grown in selective medium to midlogarithmic phase [OD_{600} = 0.8–1.0 (≈10^7 cells per ml)]. Cells to be UV- or γ-irradiated were washed once and resuspended in sterile water at a concentration of 2 × 10^7 cells per ml. Stirred cell suspensions were UV-irradiated in Petri dishes or in large culture dishes (23 × 23 cm) with a Sylvania G15T8 germicidal lamp. For γ-ray exposure, cells were irradiated with a ^{137}Cs source at a dose rate of 4 krads per min (1 rad = 0.01 gray). After irradiation, cells were washed once and resuspended at their original density in fresh medium. Cells to be treated with chemical agents were grown to midlogarithmic phase, and the relevant chemical was added directly to the medium. At selected times after irradiation or chemical treatment, aliquots of cells were withdrawn for cell density and β-galactosidase activity measurements. The doses of chemical treatments were optimized to reduce the OD_{600} of exponentially growing cultures to 70–85% of untreated cultures when measured after 4 hr of continued incubation after treatment. Heat shock treatment and amino acid starvation were carried out by protocols as described (28, 29).

RESULTS

Expression of *RAD-lacZ* Fusion Plasmids in Untreated Cells. To facilitate the construction of transcriptionally and translationally compatible *lacZ* fusions to the cloned yeast *RAD* genes of interest, we modified a *lacZ*-containing plasmid vector (24) so that one of a set of three plasmids would always generate in-frame gene fusions (see *Materials and Methods* and Fig. 1). Using productive *lacZ* fusions with *RAD1*, *RAD2*, and *RAD3*, we observed that the levels of β-galactosidase in extracts of untreated yeast cells transformed with integrating *RAD-lacZ* plasmids were lower than those observed with multicopy fusions, presumably reflecting the difference in plasmid copy number. Typical differences with *RAD2-lacZ* fusions are presented in Table 1 (0-hr treatment time), which shows that extracts of cells transformed with a multicopy plasmid contained about 80-fold more β-galactosidase activity than those from cells transformed with single-copy integrating or centromeric *RAD2* fusion plasmids. The β-galactosidase activity in untreated cells transformed with the integrating *RAD2* fusion plasmid was less

Table 1. Expression of β-galactosidase activity after treatment of cells with 4NQO

Fusion gene	4NQO	Specific activity after 4NQO treatment*						
		0	0.75	1.5	3.0	4.5	6.0	7.5
Integrated single-copy fusion plasmids								
RAD1-lacZ	−	0.23	0.20	0.19	0.23	0.31	0.25	0.24
	+	0.23	0.21	0.26	0.33	0.35	0.27	0.23
RAD3-lacZ	−	1.0	0.9	0.9	1.1	1.1	1.0	0.9
	+	1.0	0.8	0.8	1.0	1.0	0.9	1.0
RAD2-lacZ (*)	−	0.19	0.15	0.14	0.17	0.18	0.18	0.15
	+	0.19	0.50	0.52	0.38	0.35	0.32	0.32
DIN1-lacZ	−	0.17	0.16	0.17	0.25	0.28	0.30	0.24
	+	0.17	1.55	5.29	10.2	13.1	12.7	10.9
Autonomously replicating fusion plasmids								
RAD2-lacZ (*)	−	0.18	0.14	0.17	0.14	0.16	0.18	0.17
(YCp19 vector)	+	0.18	0.20	0.43	0.38	0.35	0.33	0.30
RAD2-lacZ	−	1.0	0.9	0.8	1.0	1.0	1.0	0.9
(YEp24 vector)	+	1.0	1.6	1.6	1.9	2.0	2.0	1.6

Measurement of β-galactosidase activity in extracts of cells carrying the integrated *RAD2-lacZ* fusion or single-copy centromeric plasmids (indicated by an asterisk) were carried out by using the fluorometric assay and must be divided by a factor of 15 to give values directly comparable to those obtained with the colorimetric assay.

*Specific activity is in units per cell density equivalent; hours of treatment are indicated.

than that of cells transformed with comparable *RAD3* and *RAD1* fusion plasmids by factors of about 80 and 16, respectively (Table 1).

Effect of 4NQO on Expression of β-Galactosidase. To examine the effect of DNA damage on single-copy *RAD1*, *RAD2*, and *RAD3* integrated fusions, the UV mimetic agent 4NQO was used initially because it has been shown by others that several yeast *DIN* (damage inducible) genes of unknown function are efficiently induced by this chemical (17). Yeast cells transformed with the integrating plasmid pSR16 containing a *DIN1-lacZ* fusion served as a positive control for induction and expressed β-galactosidase at levels ≈50-fold higher than those observed in untreated cells (Table 1). Extracts of cells containing *RAD1* and *RAD3* integrated fusions displayed no consistent increase in β-galactosidase activity after addition of the chemical. In contrast, a fusion gene integrated at the *RAD2* chromosomal locus reproducibly expressed 3- to 5-fold more β-galactosidase activity in treated relative to untreated cultures (Table 1). The peak of this response was typically ≈1.5 hr after 4NQO addition, whereas that for the *DIN1* fusion occurred ≈4 hr after treatment. Treatment of cells with 4NQO in the presence of cycloheximide completely abolished the response (Table 2). This result indicates a requirement for protein synthesis and suggests that the increased β-galactosidase activity reflects induction of the *RAD2-lacZ* fusion gene. A response similar to that obtained with the *RAD2-lacZ* integrant strain was observed in cells transformed with either centromeric or multicopy *RAD2* fusion plasmids containing 1350 base pairs of noncoding sequence upstream of the *RAD2* translational start codon (25). However, we reproducibly observed slightly lower levels of induction with multicopy plasmids (Table 1). No consistent differences in the kinetics of induction were noted between single-copy and multicopy plasmids carrying the *RAD2-lacZ* fusion.

Transcriptional Induction of *RAD2* by 4NQO Treatment. Total RNA and poly(A)$^+$ RNA from 4NQO-treated and untreated cells was analyzed by the method of Berk and Sharp (30) using a *RAD2*-specific DNA probe. In some experiments equal amounts of total RNA were compared (Fig. 2, lanes d–f). In others the levels of total RNA used for hybridization were adjusted to contain equal amounts of poly(A)$^+$ RNA (Fig. 2, lanes a–c) to accommodate the transient reduction in the total amount of poly(A)$^+$ RNA observed in cells exposed to DNA-damaging agents. Equal amounts of poly(A)$^+$-selected RNA were also compared (Fig. 2, lanes g–i). In all cases increased levels of *RAD2* transcript were observed in 4NQO-treated cells, with maximal levels at 30 min after treatment (Fig. 2). Induction ratios were determined from densitometric tracings of both the entire cluster of *RAD2* transcriptional start sites and the major start site. *RAD2* mRNA was induced 2.2-fold when equal amounts of total RNA were compared (average of four experiments). This value increased to 3.3-fold when normalized for fluctuations in poly(A)$^+$ RNA levels (average of five experiments). The values measured for the full spectrum of *RAD2* transcriptional start sites and for the major start site were similar in all experiments, indicating no major differences in the pattern of transcription initiation during induction. Similar experiments were carried out with these RNA preparations using *RAD4*-specific (R. Fleer and E.C.F., unpublished data) and *RAD10*-specific (9) DNA probes. There was no evidence of enhanced levels of transcripts of these genes (data not shown).

Induction by Other DNA-Damaging Agents. We examined the levels of β-galactosidase activity in cells containing an integrated *RAD2-lacZ* fusion after treatment with a variety of DNA-damaging agents. Induction of the *RAD2-lacZ* fusion occurred at higher doses of UV-radiation than did the

Table 2. Induction of integrated single-copy *RAD2-lacZ* fusion gene by various agents

Agent	Dose	Induction ratio*
None	—	1.0
UV-radiation	35 J/m^2	5.2 (1.5)
γ-Rays	40 krad	4.9 (1.5)
Nalidixic acid	50 μg/ml	3.6 (1.5)
4NQO	0.25 μg/ml	3.4 (1.5)
4NQO/CH	0.25 μg/ml (50 μg/ml)	0.7
Bleomycin	100 μg/ml	2.5 (3)
Nitrogen mustard	0.2 mM	2.4 (3)
Mitomycin C	100 μg/ml	2.3 (3)
MeMes	0.02%	1.9 (3)
Methotrexate	10 μg/ml	2.0 (6)
Heat shock	23°C → 37°C	1.0
Amino acid starvation	—	0.5

CH, cycloheximide; MeMes, methyl methanesulfonate.

*The induction ratio (peak activity) is the ratio of β-galactosidase activity in treated to untreated cells; numbers in parentheses are the time (in hours) of peak activity.

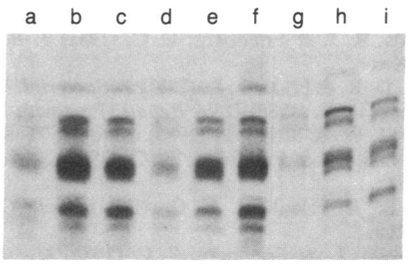

FIG. 2. S1 nuclease analysis of uninduced and induced *RAD2* transcripts. The entire cluster of *RAD2* transcriptional start sites (25) is shown. Lanes: a–c, total RNA was adjusted for poly(A)$^+$ content, and the lanes contained 100, 147, and 114 μg of RNA, respectively; d–f, equal amounts of total RNA (100 μg) were present in each lane; g–i; 40 μg of poly(A)$^+$ RNA from a separate preparation was in each lane. Each set of three lanes represents exposure of cells to 4NQO for 0, 30, and 60 min (from left to right). The *RAD2* DNA probe used was a 1.4-kilobase *Eco*RI–*Sal* I fragment (25) end-labeled at the *Eco*RI site.

DIN1-lacZ fusion and reached a maximum level of ≈6-fold (Fig. 3*A*). Comparable levels (3- to 6-fold) of induction were observed after treatment of cells with γ-radiation or nalidixic acid (Table 2). [The site of action of nalidixic acid in yeast is not clear. The related compounds novobiocin and oxolinic acid do not inhibit yeast DNA topoisomerase II (31). Recent studies with *E. coli* suggest that nalidixic acid may bind directly to DNA (32).] Lower levels of induction (≈2-fold) were reproducibly observed after treatment with the alkylating agents methyl methanesulfonate or nitrogen mustard or with bleomycin, mitomycin C, or methotrexate (Table 2). No induction of the *RAD2-lacZ* fusion was observed in cells exposed to heat shock or to amino acid starvation.

As with 4NQO, a single dose of UV-radiation led to a rapid increase in β-galactosidase activity, with a peak at ≈1.5 hr, and then decreased to a lower plateau level (Fig. 3*B*). On the other hand, after treatment of cells with nitrogen mustard, β-galactosidase activity increased gradually and peaked later, at ≈3 hr (Fig. 3*B*). Most treatments induced the *RAD2* gene with kinetics similar to those after treatment with either UV-radiation or nitrogen mustard (Table 2). However, methotrexate, which interferes with normal DNA replication, resulted in a maximum level of induction ≈6 hr after treatment. A consistent finding was that agents that induced the *RAD2-lacZ* fusion relatively rapidly (i.e., with a peak response at ≈1.5 hr) elicited a greater maximal level of induction than did those that induced it with slower kinetics (Table 2).

DISCUSSION

There is no evidence in any eukaryotic system for a regulon comprising multiple genes, all of which respond to DNA damage or arrested DNA replication and are under the control of a single repressor analogous to the lexA protein of *E. coli*. Nonetheless, there are indications of enhanced expression of gene functions in both higher and lower eukaryotes after exposure of cells to DNA damage (for recent reviews, see refs. 4 and 33–36). In most of these instances, the genes involved are undefined; however, in some reports specific yeast genes have been identified as damage-inducible. The *RAD54* gene is required for genetic recombination in yeast (1), and survival of a temperature-sensitive *rad54* mutant after γ-irradiation is dependent on active protein synthesis at the permissive temperature (37). Peterson *et al.* (38) demonstrated enhanced expression of the *CDC9* gene (which encodes DNA ligase) after exposure of cells to UV-radiation, and McClanahan and McEntee (39) and Ruby and Szostak (17) independently showed the induction of multiple genes of unknown function after treatment of yeast cells with UV-radiation, γ-rays, 4NQO, methotrexate, or alkylating agents. Comparisons of the restriction map of the *RAD2* gene with those of *DIN1*, *DIN3*, *DIN4*, and *DIN6* showed no overlap.

None of the examples quoted above are analogous to known inducible genes in *E. coli*. In contrast, the *RAD2* gene is a member of a class of yeast excision-repair genes that are formally analogous to the *uvr* excision-repair genes in *E. coli*, some of which (*uvrA*, *uvrB*, and *uvrD*) are inducible by DNA damage (4, 5, 40). Under experimental conditions that include the use of the DNA-damaging agents UV-radiation, 4NQO, and γ-rays, as well as nalidixic acid, we reproducibly observed an ≈3- to 6-fold increase in the β-galactosidase activity of cells containing a single integrated copy of a *RAD2-lacZ* fusion gene. This phenomenon apparently does

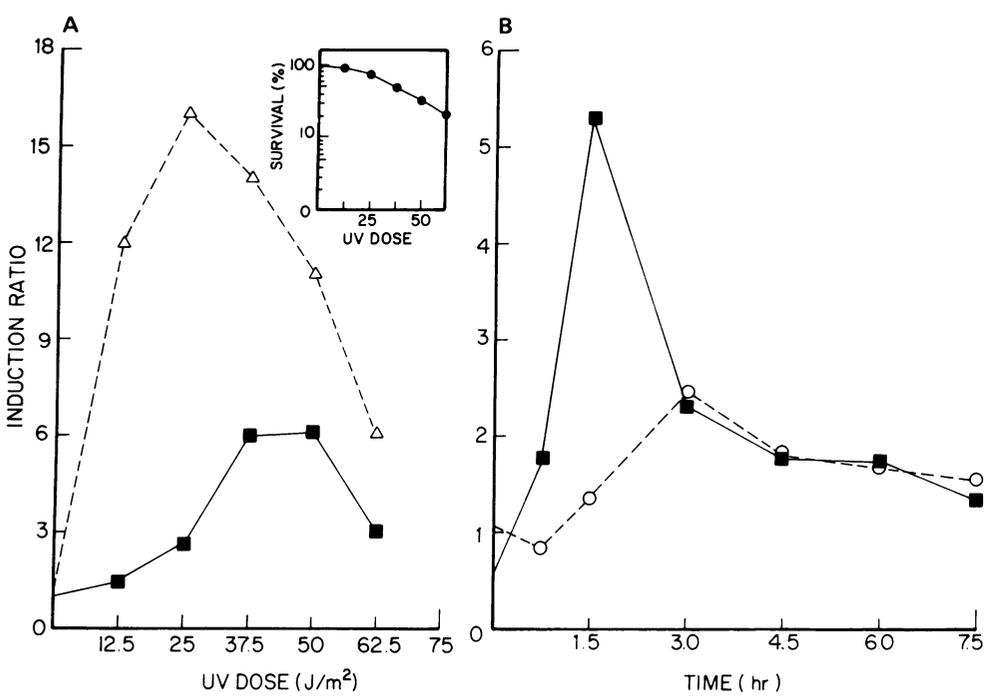

FIG. 3. (*A*) Yeast strains containing integrated *DIN1-lacZ* (△) or *RAD2-lacZ* (■) fusions were UV-irradiated and assayed for β-galactosidase 4 hr or 1.5 hr later, respectively. (*Inset*) Survival was identical for both strains (●). (*B*) A strain containing an integrated *RAD2-lacZ* fusion was untreated or treated with either 35 J/m^2 of UV-radiation (■) or 0.2 mM nitrogen mustard (○) and assayed for β-galactosidase at various times. The induction ratio is the β-galactosidase specific activity measured in treated cultures divided by that measured in untreated control cultures.

not simply represent the response to damage of a very small population of surviving cells because, in the case of UV irradiation, it was directly demonstrated that the majority of the cells were viable. In the case of 4NQO treatment, we have shown that this enhanced expression is reflected at the transcriptional level and is strictly dependent on active protein synthesis. Thus, it likely represents enhanced synthesis of new Rad2 protein rather than modification of existing protein. Induction occurred to a lesser extent (≈60%) with the DNA damaging agents nitrogen mustard, mitomycin C, and bleomycin and after treatment of cells with methotrexate.

It remains to be established whether or not the *RAD2* gene and other inducible yeast genes are under the control of a common regulatory system. In *E. coli* there is evidence that, in addition to the SOS pathway, at least two other regulated sets of genes respond to DNA damage (40). One of these involves genes that are regulated by the *ada* locus and are induced after exposure of cells to agents that alkylate the O^6 position of guanine in DNA. A second example involves the induction of the *groEL* and *dnaK* heat shock genes in response to certain DNA-damaging agents. The induction of both of these genes is apparently controlled by the *htpR* gene of *E. coli*.

Besides the functional analogy between the yeast *RAD* genes and the *E. coli uvr* gene, our studies suggest other parallels between induction of the yeast *RAD2* gene and the *E. coli* SOS response. The magnitude of the induction of the *uvrA*, *uvrB*, and *uvrD* genes of *E. coli* (≈5-fold) is very similar to that observed with *RAD2*. In addition, both organisms demonstrate differences in the kinetics of the response to different inducing treatments. In *E. coli* it has been suggested that the inducing signal for SOS induction may be related to DNA degradation (41), and it has been pointed out (41) that agents that directly promote DNA strand breakage result in early induction, whereas those that cause base damage requiring enzyme-induced DNA degradation (e.g., pyrimidine dimers) cause induction at later times. Agents that inhibit DNA replication (associated with late degradation of DNA) cause the slowest induction. In the present study, similar kinetic distinctions were observed during the induction of *RAD2*, although the parallel with *E. coli* is not absolute. Thus, in *E. coli* bleomycin causes rapid induction of the SOS response relative to UV-radiation, while the reverse is observed for *RAD2*. It is not clear to what extent these results reflect differential access to yeast DNA of the various chemical agents tested and/or their relative stability in solution.

The sequences necessary for the induction of the *RAD2* gene are apparently located within 1350 base pairs of the translational start site.

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