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Specific *Saccharomyces cerevisiae* Genes Are Expressed in Response to DNA-Damaging Agents

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When exposed to DNA-damaging agents, the yeast *Saccharomyces cerevisiae* induces the expression of at least six specific genes. We have previously identified one damage inducible (*DIN*) gene as a gene fusion (*din-lacZ* fusion) whose expression increases in response to DNA-damaging treatments. We describe here the identification of five additional *DIN* genes as *din-lacZ* fusions and the responses of all six *DIN* genes to DNA-damaging agents. Northern blot analyses of the transcripts of two of the *DIN* genes show that their levels increase after exposure to DNA-damaging agents. Five of the *din-lacZ* fusions are induced in *S. cerevisiae* cells exposed to UV light, gamma rays, methotrexate, or alkylating agents. One of the *din-lacZ* fusions is induced by either UV or methotrexate but not by the other agents. This finding suggests that there are sets of *DIN* genes that are regulated differently.

Procaryotic and eucaryotic cells respond to the stresses caused by DNA damage or inhibited DNA replication in numerous ways. In *Escherichia coli* three inducible systems have been characterized: the SOS response, which is quickly activated after exposure to many DNA-damaging agents and replication inhibitors (53); the adaptive response to alkylation, which is elicited by certain alkylating agents (6, 43); and the adaptive response to oxidative damage such as that caused by hydrogen peroxide (12).

The SOS response involves a number of diverse functions including increased recombination and DNA repair, enhanced mutagenesis, *recA* protein synthesis, prophage induction, Weigle reactivation of irradiated phage, colicin production, and filamentation. At least 17 genes, some of which have been identified with specific functions, are induced during this response. These genes are coordinately regulated by the *recA* and *lexA* gene products (28, 52). The two adaptive responses result in decreased lethality caused by particular types of DNA damage. They appear to operate independently of the SOS response as they are neither under the control of the *lexA* and *recA* genes nor induced by many SOS-inducing agents. The adaptive response to alkylating agents also leads to decreases in mutagenesis by such agents as ethyl methane sulfonate (EMS) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (27). Although these three responses seem to be distinct, there may be some interaction among them as evidenced by the fact that at least two SOS genes are induced by MNNG (2, 39).

Analogous inducible responses to DNA-damaging agents may be present in both lower and higher eucaryotes. The existence of an inducible DNA repair system in *Neurospora crassa* is suggested by the finding that cells exposed to sublethal doses of UV, X ray, or nitrous acid have an enhanced ability to rescue lethally irradiated cells when fused into a heterokaryon after treatment (49). Baker (3) used a dimer specific endonuclease assay to demonstrate both constitutive and inducible components of excision repair in *N. crassa*. An inducible component of UV-damage repair in *Saccharomyces cerevisiae* is detectable in split-dose experiments (13, 46). In both *Ustilago maydis* (24) and *S. cerevisiae* (5) the inhibition of protein synthesis by cycloheximide immediately after gamma irradiation blocks the repair of double-strand breaks and decreases cell survival. An inducible pathway of recombination would explain the increased frequency of intragenic recombination in unirradiated diploid yeast cells after mating with X-irradiated haploids (14). This induction of recombination in the unirradiated nucleus is seen even when nuclear fusion is blocked.

A large number of genes in *S. cerevisiae* are involved in repair, recombination, or both (16, 26). Most of these genes have been identified and studied by the isolation and characterization of mutants defective in repair, recombination, or both. These mutants often have increased sensitivity to radiation, DNA-damaging chemicals, or both. Some of the genes have been assigned to one of three epistasis groups that are named for one locus in each group; *RAD3*, *RAD6*, and *RAD52*. Genes of the *RAD3* group are primarily involved with pyrimidine dimer excision, whereas genes of the *RAD52* group are required for double-strand break repair. Mutations in genes of the *RAD6* group are pleiotropic; for example, mutations in *rad6* lead to decreased mutagenesis, increased spontaneous recombination, and defective sporulation.

The presence of damage-inducible functions and the existence of recombination and repair mutants suggests that yeasts, like *E. coli*, have many coordinately regulated DNA-damage-inducible genes. We have described previously a method for cloning regulated yeast genes (42). In this method, random yeast genes are fused to the *E. coli lacZ* gene in vitro. The fusions are made on a vector containing a truncated *lacZ* fragment (9) that lacks the *lac* promoter and the codons for the first eight amino acids of beta-galactosidase. Expression of the *lac* fragment in yeast cells requires the insertion of a promoter and a translational start site and results in the production of a hybrid protein with beta-galactosidase activity. The vector contains a unique restriction endonuclease site at the 5' end of the *lacZ* fragment, into which random fragments of yeast DNA can be inserted. The

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ligated DNA is used to transform yeast cells to create a library of fusions, and the transformants are replica plated onto 5-bromo-4-chloroindolyl-β-D-galactoside (Xgal) indicator plates to screen for productive fusions. We screened such a library for fusions induced by DNA-damaging agents and identified the *din1-lacZ* fusion.

We report here that the isolation of the five additional *din* gene fusions from *S. cerevisiae*, the characterization of these genes, and their responses to various DNA-damaging agents. Our results show that several *S. cerevisiae* genes are specifically induced by DNA-damaging agents and suggest that there are at least two sets of *DIN* genes that are regulated differently.

MATERIALS AND METHODS

Strains. *S. cerevisiae* A2 (α *his3-11,15 leu2,112*) was originally strain LL20 from L. Lau. Strain A15 (α *gal mal*) was originally strain S288C. Strain A110 (**a** *met10-3/met10-5 trp1 ade1*) which was disomic for chromosome VI was a gift from R. Mortimer. Strain T378 is strain A2 transformed with pSZ214 containing the *din1-lacZ* fusion (42). Diploid strain DA121 (**a**/α *his3-11,15/HIS3 leu2-2,112/leu2-2,112 met10/MET10 trp1/TRP1 ura3/URA3 tcm1/TCM1*) has a single copy of the fusion plasmid A4P4 (*his3-lacZ LEU2*) (42) integrated at the *his3* locus. Haploid strain TSR40-9 (α *his3-11,15 leu2,112 tcm1*) has the fusion plasmid A30p2 (*his3-lacZ TRP1*) containing the *ars1* sequence. Strains DA121 and TSR40-9 were gifts from A. Murray. Strain D741-3B (**a** *leu2-2,112 his3-11,15 trp1 ura3*) was constructed by crosses of strain A2 with strains A110, D234-3B (obtained from P. Brown), and T378 to obtain a strain with 95% A2 background. Strains TSR30-15 and TSR31-39 were obtained by transformation of strain D741-3B with plasmids pSR16 and pSR18, respectively (see Fig. 2). All other *S. cerevisiae* strains are described in the text.

E. coli 5346 (Leu$^-$ Thr$^-$ Thi$^-$ HsdR$^-$ HsdM$^-$), HB101, and JA300 (Thr$^-$ Leu$^-$ Thi$^-$ Trp$^-$ Thy$^-$ HsdR$^-$ HsdM$^-$ Str^r) were used for plasmid isolation and propagation.

Chemicals and media. Redistilled methyl methane sulfonate (MMS) was a gift from F. Sherman. EMS was from Eastman Kodak Co. 4-Nitroquinoline-*N*-oxide (NQO) (Sigma Chemical Co.) was dissolved in ethanol-acetone (8:1) at 0.5 mg/ml and stored at 0°C. MNNG which was a gift from P. Foster and E. Eisenstadt was originally obtained from ICN Pharmaceuticals Inc. Aliquots of a solution (2.5 mg/ml of 20 mM acetate buffer [pH 5.5]) were stored at −20°C and used only once after thawing on ice.

Synthetic selective and YPD media were those of Sherman et al. (45). Buffered synthetic selective medium supplemented with Xgal was as described previously (42). Thymine starvation plates and NQO plates contained buffered synthetic selective medium supplemented with Xgal (40 μg/ml) and either methotrexate (35 μg/ml) and sulfanilamide (5 mg/ml) or NQO (0.05 μg/ml), respectively. Methotrexate and sulfanilamide were from Sigma Chemical Co.

Fusion libraries and screenings. The creation of the random yeast gene fusions and the libraries of yeast transformants containing the fusions have been described previously (42). The fusions were made in vitro by ligating *S. cerevisiae* DNA partially digested with *Sau*3A enzyme to *Bam*HI-cleaved vector pSZ211 DNA. This vector contains the yeast *LEU2* and *ars1* sequences; the pBR322 beta-lactamase and ori sequences for selection and replication in *S. cerevisiae* and *E. coli*, respectively; and a truncated *lacZ* fragment. Library 1 contained nearly 20,000 transformants from a single transformation pooled into 20 groups. Each cell pool was stored in 50% glycerol at −70°C. Library 2 contained 8,945 independent colonies from a transformation different from that used to generate library 1.

To screen for the *din2-lacZ* fusion, ca. 4,000 transformants from 19 of the library 1 pools were grown on synthetic selective medium without leucine and then replica plated onto Xgal plates. (Pool 1 was not screened because the *din1-lacZ* fusion was identified in it.) After 1 week of incubation at 30°C, transformant colonies expressing beta-galactosidase were picked, grown on selective medium, and replica plated to control Xgal plates, Xgal plates containing 0.05 μg of NQO per ml, and Xgal plates which were subsequently exposed to a UV dose of 16 J/m^2. The colony T671 containing this fusion was darker blue on treated plates than on control plates at ca. 48 h after replica plating.

To screen for the *din3* to *din6* fusions, the colonies from library 2 were patched onto master plates of selective medium, incubated for 2 days at 30°C, and replica plated onto control and methotrexate Xgal plates as well as onto Xgal plates that were subsequently exposed to either 16 J of UV per m^2 or 50 krads of gamma irradiation. Those plates which were to be gamma or UV irradiated were incubated for 4 to 6 h before treatment. All replicas were incubated at 30°C. After 2 to 7 days, the strains of interest were streaked onto selective medium plates to obtain pure colonies for additional tests.

The fusions identified in the above screenings were further tested by quantitative assays of induced and uninduced levels of beta-galactosidase in the *S. cerevisiae* transformants grown in liquid culture. Cells at mid-log phase in seletive medium (without leucine) were either treated with 16 J of UV per m^2 or given 0.01% MMS and subsequently shaken at 30°C. At every 2 h during a 10-h incubation, culture samples were assayed for cell density and beta-galactosidase activity as described below.

S. cerevisiae transformants were assayed for the mitotic stability of the plasmid *LEU2* marker as previously described (34).

Irradiation conditions. Colonies were gamma irradiated at 23°C with a ^{60}Co source (School of Public Health, Harvard Medical School) at a dose rate of 3.7 krads/min for a total dose of 50 krads (50% survival). Colonies or cell suspensions were UV irradiated with a Sylvania G15T8 germicidal lamp which was calibrated with a model IL254 International Light photometer. Cell suspensions were washed three times with water and resuspended at 2×10^7 cells per ml of water before irradiation. The cells were continually stirred during irradiation in a petri dish (1 ml/3.7 cm^2), after which they were filtered and resuspended at ca. 1×10^7 cells per ml of fresh medium. Suitable precautions were taken to prevent photoreactivation.

Beta-galactosidase assays. *S. cerevisiae* transformants were grown in synthetic selective medium (without leucine or uracil). Mid-log-phase cultures (optical density at 600 nm of 1.0 and 10^7 cells per ml) were treated by the addition of MMS, EMS, MNNG, or NQO directly to the medium or by UV irradiation at the concentrations or doses described in the text. At various times after treatments or additions, the cultures were monitored for cell density (optical density at 600) and for beta-galactosidase activity. Initially we permeabilized cells with chloroform and sodium dodecyl sulfate and immediately thereafter assayed enzyme activity with *o*-nitrophenyl-β-D-galactopyranoside as the substrate as previously described (42). Later (all data presented here were derived from these assays unless otherwise indicated) we

disrupted the cells by vortexing them in the presence of 0.5 g of acid-washed glass beads (diameter 0.45 μm; Sigma Chemical Co.) in a total volume of 0.8 ml of Z buffer containing 0.01% sodium dodecyl sulfate and 300 μM phenylmethylsulfonyl fluoride. Although both types of cell preparations gave similar results, we found that the disrupted cells gave more reproducible results than the permeabilized cells for cells induced to high beta-galactosidase levels. Beta-galactosidase activities are expressed as specific activities in units (31) normalized to cell density.

DNA extractions, plasmid constructions, and restriction site mapping. Conditions for yeast and plasmid DNA minipreparations, DNA ligations, fusion plasmid isolations, and screenings of fusion expression in *E. coli* were as described previously (42). Large-scale plasmid preparations (10) were purified on cesium chloride gradients. Conditions for the restriction endonucleases were those of the supplier New England Biolabs.

The fusion plasmids pSR1, pSR10, and pSR25 from the *S. cerevisiae* transformants T8b, T16b, and T22b, respectively, were cloned in bacteria by digesting yeast DNA from each strain with a restriction endonuclease, ligating the DNA in a dilute solution to circularize the plasmids, transforming *E. coli* 5346, and selecting for ampicillin-resistant bacteria. Bacterial transformants were not obtained when uncut yeast DNA was used. The restriction enzymes were chosen on the basis of preliminary mapping of the fusions in the *S. cerevisiae* strains by Southern blot analyses with a *lacZ* probe (data not shown): *Xho*I for plasmid pSR1, *Bam*HI for plasmid pSR25, and *Hin*dIII for plasmid pSR10. Most transformants were Lac$^+$ Leu$^+$. Their plasmids were examined by restriction mapping by standard techniques (29). The cloning of plasmid pSZ214 by transformation with *Hin*dIII-cut yeast DNA has been described previously (42).

The subcloning of fusion fragments into pBR322 or pMC1403 to generate *din* probes involved standard cloning techniques. The *Hin*dIII-*Sal*I fragments containing the *lacZ* fragment and the adjacent yeast inserts from plasmids pSR1 and pSR3 (see Fig. 2) were ligated to *Hin*dIII-*Sal*I-cut pBR322 to generate the ampicillin-resistant plasmids pSR8 and pSR10, respectively. Similarly, the *Sal*I fragment from pSZ214 was cloned into the *Sal*I site of pBR322 to obtain pSR7. The *Bgl*II-*Sac*I fragment encompassing the yeast insert and the 5′ portion of the *lacZ* sequence in pSR25 was ligated to *Bam*HI-*Sac*I-cleaved pMC1403 to create pSR36. Plasmids pSR7, pSR8, pSR10, and pSR36 produced beta-galactosidase activity in *E. coli* HB101.

An integrating *lacZ* fusion vector, pSR14, containing the *S. cerevisiae URA3* selectable marker, pBR322 *ori* and *amp*^{r} sequences, and the truncated *lacZ* fragment was constructed with fragments from three different plasmids. The 2.6-kilobase (kb) *Pvu*II-*Sal*I fragment from pJT29 contained the 1.1-kb *URA3* fragment which had been cloned into the pBR322 *Ava*I site by dGdC tailing. Plasmid pJT29 was a gift from Jim Thomas. The 6.3-kb *Bam*HI-*Sal*I fragment containing the truncated *lacZ* fragment was obtained from pMC1403. The 2.7-kb *Pvu*II-*Bam*HI fragment contained the pBR322 *ori* and *amp*^{r} sequences. These fragments were electrophoresed on agarose gels, eluted, ligated, and used to transform bacterial strain MB1000 to ampicillin resistance. The structure of the plasmid from an *amp*^{r} *ura*$^+$ colony was confirmed by restriction mapping. Plasmid pSR15 was a derivative of pSR14 in which the single *Eco*RI site was destroyed by a fill-in reaction with T4 polymerase (29). The integrating plasmids pSR16 and pSR18, containing the *din1* and *din3* fusions, respectively, were constructed by ligating the 3-kb *Bam*HI-*Sac*I fragment from pSZ214 and the 2.8-kb *Hin*dIII-*Sac*I fragment from pSR8 to the appropriately cleaved pSR15 DNA. The plasmids were cloned by transformation of strain MB1000 to ampicillin resistance.

We generated the restriction site maps of the genomic *DIN* genes by using data from Southern blots of strain A15 DNA probed with *din* fusion probes. The DNA (2 μg per sample) was cut with restriction endonucleases as described in the text, fractionated by size on 0.5 or 0.7% agarose gels, and then transferred to either nitrocellulose (48) or diazobenzyloxymethyl-paper (1). The *din* fusion probes as described in the text were restriction fragments that were extracted from agarose by electroelution onto DEAE paper or from low-melt agarose by phenol (29) and subsequently labeled by nick translation (40). The conditions for transfer and hybridization to nitrocellulose or diazobenzyloxymethyl paper blots were those used by Orr-Weaver et al. (35) or Alwine et al. (1), respectively. Autoradiography was performed with XRP-5 Kodak film at −70°C in the presence of an intensifying screen. Labeled pSZ63 plasmid DNA (containing the *HIS3* gene [33]) was subsequently hybridized to the blots to monitor the completeness of the restriction endonuclease digestions of the A15 DNA. The *HIS3* gene and adjacent sequences have been mapped with restriction endonucleases (50).

RNA extraction and analysis. MMS (0.01%) was added to one portion of mid-log-phase strain A2 cells in YPD medium, and the other portion was left untreated. During subsequent growth at 30°C, culture samples were quickly cooled on ice and pelleted by centrifugation, and total RNA was extracted with phenol from cells disrupted by vortexing in the presence of glass beads (17). Total RNA (10 μg per lane) was fractionated by size on formaldehyde denaturing agarose gels (25), transferred to nitrocellulose, and hybridized with the ^{32}P-labeled probes described in the text. The conditions for transfer and hybridization were as described previously (42). Vesicular stomatitis virus RNAs labeled in vivo were used as molecular weight markers. Band density on the autoradiograms was measured with a Helena Laboratories Quick Scan densitometer.

RESULTS

Isolation of *din-lacZ* fusions. The *din2-* to *din6-lacZ* fusions were identified by screening libraries of random gene fusions in *S. cerevisiae* for those yielding beta-galactosidase activity inducible by DNA-damaging treatments. Previously we had constructed two separate libraries of fusions in yeasts (42). These two libraries were screened differently. Library 1 was screened by replica plating tests on control Xgal plates, Xgal plates containing NQO, and Xgal plates that were subsequently exposed to UV. The *din2-lacZ* fusion was identified in a colony (transformant T671) that was darker blue on UV and NQO plates than on control plates. The screening for this fusion was similar to that used for the *din1-lacZ* fusion. Library 2 was screened by replica plating tests with three different DNA-damaging agents to identify *din* fusions that might be induced only by specific agents. The three agents, methotrexate, UV, and gamma rays, differ as to the kinds of lesions they generate (21, 23). Of the 8,945 independent transformants in the library, 20 were darker blue on any two of the treated Xgal plates than on control Xgal plates. Of these 20 transformants, 4 (T8b, T16b, T22a, and T22b) containing the *din3*, *din4*, *din5*, and *din6* fusions, respectively, gave inducible beta-galactosidase activity in subsequent quantitative assays as described above (data not shown).

TABLE 1. *din1-lacZ* fusion induction by UV or MMS[a]

Agent	Dose[b]	U of beta-galactosidase	Fold increase
None		0.20	
UV	4	3.1	15
	8	8.0	40
	16	19	93
	25	22	110
	34	15	76
	49	3.8	19
None		0.24	
MMS	0.0025	49	200
	0.005	74	310
	0.01	52	220
	0.025	38	160
	0.05	3	15
	0.1	0.21	

[a] Mid-log-phase haploid *S. cerevisiae* T378 was either treated once with UV and subsequently grown for 4 h or grown in MMS for 4 h. Each value is the mean of three determinations from a single culture.

[b] Doses are measured in joules per square meter for UV and in percentages for MMS.

When we examined all six *din-lacZ* fusions in parallel on Xgal plates, we found that five of the six *din-lacZ* fusions were induced by UV, gamma rays, or methotrexate, whereas one fusion, *din5*, was induced by UV or methotrexate but not by gamma rays.

Induction kinetics. The responses of the six *din* fusions were further studied by quantitatively assaying beta-galactosidase in cells grown in liquid culture. Their expression depends on the dose and type of the DNA-damaging agent, as well as on the time after the initial treatment. We studied the effects of either UV or MMS, an alkylating agent that has many of the same effects in yeast cells as do gamma rays (23). The *din1-lacZ* fusion was studied in greatest detail. It is strongly induced by both MMS and UV (Table 1). The optimal doses for induction have only slight effects on cell viability and growth: for example, 80% of the cells were viable after a UV dose of 16 J/m^2, and cells grown in 0.01% MMS were at 80% of the control cell density 4 h after the addition of MMS (generation time, ca. 2 h).

The time courses of the responses of the *din-lacZ* fusions to DNA-damaging agents were determined. Beta-galactosidase activity was measured in log-phase cells after either a single UV treatment with 16 J/m^2 or the addition of 0.01% MMS to the growth medium. These doses are optimal for *din1-lacZ* induction (Table 1). Representative data from all six *din* fusions are shown in Fig. 1. Five of the six fusions (*din1*, *din2*, *din3*, *din4*, and *din6*) have similar induction kinetics; beta-galactosidase specific activity increases within 2 h after UV irradiation or MMS addition and continues to increase up to 6 to 8 h, at which time the cells enter stationary phase. They also are induced by both UV and MMS. The *din1*, *din2*, *din3*, and *din6* fusions all show a two- to threefold higher induction by MMS than by UV (Fig. 1 and Table 1). The *din4-lacZ* fusion is induced to approximately the same levels by either UV or MMS (fourfold induction). In striking contrast to these five fusions, the *din5* fusion is induced by UV but not by MMS. The beta-galactosidase activity in UV-irradiated cells is slightly higher than in control cells at 4 h, increases up to 6 h, and then declines (Fig. 1). MMS causes no significant increases in beta-galactosidase levels above control levels for the *din5* fusion even at 10, 12, and 24 h after the addition of MMS.

The vast majority of yeast gene *lacZ* fusions are not induced by DNA-damaging agents. Seventeen other productive fusions from the two libraries show no significant increases in beta-galactosidase activity under these conditions (data not shown). Finally, two strains containing a *his3-lacZ* fusion either stably integrated into chromosomal DNA or on an autonomously replicating plasmid show no increases in enzyme activity greater than twofold when treated with either MMS or UV (Table 2).

Induction by other DNA-damaging agents. Because the *din5-lacZ* fusion is not induced by MMS or gamma rays, the effects of several different DNA-damaging agents (UV, NQO, MMS, EMS, and MNNG) on all the *din* fusions were examined. NQO is a potent mutagen whose effects in *S. cerevisiae* mimic those of UV (37). EMS and MNNG are alkylating agents. The doses used give maximum induction of the *din1-lacZ* fusion and only slightly inhibit cell growth; the treated cultures grow to ca. 80% of the control cell density at the time of assay, 5 h after the start of treatment. The results of two experiments in which either permeabilized or disrupted cells were used for beta-galactosidase assays are summarized in Table 2. All the fusions except *din5* are induced strongly by UV, NQO, MMS, and MNNG. EMS induces *din1*, *din3*, *din4*, and *din6* but does not affect *din2* and *din5*. The *din5-lacZ* fusion is induced by UV but not by the other agents. Thus the fusions could be grouped

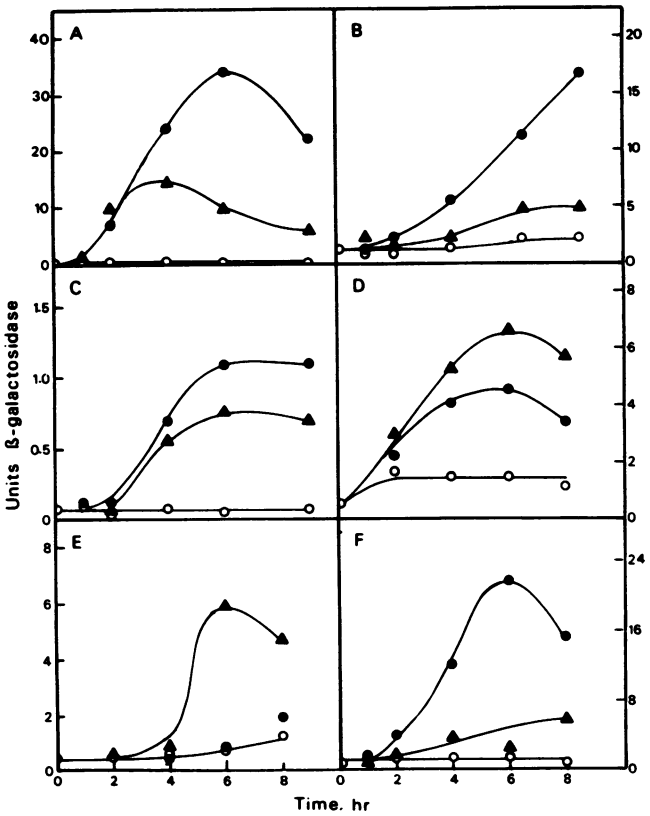

FIG. 1. Induction kinetics of the *din-lacZ* fusions. Haploid *S. cerevisiae* transformants containing the *din* fusions were grown to mid-log phase in selective medium. At 0 h, portions of the cultures were given 0.01% MMS (●), treated with 16 J of UV per m^2 (▲), or left untreated as a control (○). During further growth at 30°C, samples were removed and assayed for cell density and beta-galactosidase activity. Shown are the results for the *din* fusions: A, *din1*; B, *din2*; C, *din3*; D, *din4*; E, *din5*; F, *din6*.

into two classes according to their responses to alkylating agents: the *din5* fusion is induced by UV but not by alkylating agents, whereas the others are induced by many agents including alkylating agents.

The *din-lacZ* fusions also differ from each other both in the basal levels of enzyme produced and in the maximum induction ratios attained (Fig. 1 and Table 2). Fusions 1 and 3 have the lowest basal levels (mean values of 0.3 and 0.1 U, respectively) and the highest induction ratios (100- to 300- and 20- to 40-fold, respectively, with most agents) of the six fusions. The four other fusions generally have higher basal levels and lower induction ratios (from 4 to 10); however, we did observe a variation in these characteristics for the *din5* fusion (see below). These differences among the fusions could be due to differences in *DIN* gene expression, in plasmid stability or number, in fusion transcript or protein stability, or in some other property.

Expression of *din-lacZ* fusions reintroduced into *S. cerevisiae*. To determine whether plasmid stability or copy number could account for the differences in *din* fusion expression, we first analyzed the mitotic stabilities of the fusion plasmids in the original yeast transformants. Because the fusion plasmids contain a sequence for replication in yeast cells, they are capable of either autonomous replication or chromosomal integration. Integrated plasmids are normally quite stable mitotically and are maintained at a low copy number, whereas autonomously replicating plasmids are quite unstable and are maintained at a high copy number. The strongly induced *din-lacZ* fusions *1* and *3* were on mitotically stable plasmids, whereas the others were on unstable plasmids. A mitotically stable derivative of the *din5-lacZ* fusion was isolated during strain passage. This derivative has a lower basal level of expression and a higher induction level (Table 2) than the unstable parent (Fig. 1).

We next cloned the fusion plasmids in *E. coli* as described below, reintroduced them into haploid yeast cells, and studied their expression. When the *din1-lacZ* fusion plasmid (pSZ214 in Fig. 2) is autonomously replicating, the beta-galactosidase basal level in yeast cells is lower than when the plasmid is integrated (0.04 versus 0.2 U). The induction ratio, however, is the same in both cases (an average of 300-fold). When the *din2* and *din5* fusions are reintroduced into *S. cerevisiae* on replicating plasmids, their basal levels and induction ratios are the same as in the original unstable transformants. Furthermore, the *din5* fusion in the new strain again responds to UV but not to MMS (data not shown). Thus the copy number and plasmid stability could be responsible for the differences in levels of expression of a fusion, but they do not account for the differences in the types of agents to which the fusions respond.

To compare the regulation and expression of the fusions in a more controlled manner, we constructed strains with single or multiple copies of a *din* fusion integrated into chromosomal DNA. Two fusions were cloned into a different *lacZ* fusion vector that propagates in *S. cerevisiae* only by integrating into genomic DNA. Each of the two plasmids (plasmids pSR16 and pSR18 in Fig. 2) was targeted to integrate into its homologous *DIN* locus in haploid *S. cerevisiae* transformants with the technique developed by Orr-Weaver et al. (34). The position and copy number of each integrated fusion in representative transformants were confirmed by Southern blot analyses (data not shown). The yeast transformants containing single or multiple integrated copies of either the *din1* or *din3* fusion were assayed for their beta-galactosidase activities when treated with MMS or when left untreated. These two *din* fusions give nearly the same basal levels and induction ratios in these new transformants as compared with the transformants containing the original plasmids (Tables 2 and 3).

Structures of the *DIN* genes and fusions. The *din-lacZ* fusions were cloned in *E. coli* so that their structures could be characterized and *DIN* gene specific hybridization probes could be generated. Since the fusions were originally constructed on a shuttle vector, *E. coli* transformants containing the *din-lacZ* fusions were obtained by transformation with DNA (either uncut, or cut with a restriction endonuclease and then ligated as detailed above) from each yeast strain and by selection for ampicillin resistance. The plasmids were

TABLE 2. Fusion induction by different DNA-damaging agents[a]

Expt	Fusion	U of beta-galactosidase induced by:					
		No agent	UV	NQO	MMS	EMS	MNNG
A	*his3a*	2.7 ± 0.10	2.3 ± 0.37	ND[b]	3.9 ± 0.51	ND	2.7 ± 1.2
	his3b	5.2 ± 0.12	5.7 ± 0.27	5.1 ± 0.29	6.1 ± 0.72	4.7 ± 0.81	6.2 ± 0.89
B	*din1*	0.4	52	54	ND	ND	38
	din2	1.5	3.8	7.9	ND	1.8	8.3
	din3	0.21	5.1	0.92	ND	0.9	1.2
	din4	2.5	11	12	ND	13	13
	din5	ND	ND	ND	ND	ND	ND
	din6	1.1	3.4	4.5	ND	3.7	3.6
C	*din1*	0.16	19	7.9	ND	4.2	16
	din2	ND	ND	ND	ND	ND	ND
	din3	0.07	2.7	0.51	2.9	0.27	2.2
	din4	2.8	5.8	4.1	5.8	3.2	4.2
	din5	0.02	1.6	0.03	0.03	0.03	0.03
	din6	0.54	ND	1.0	3.2	0.71	1.3

[a] At 5 h after treatment either glass bead cell extracts (experiments A and B) or permeabilized cells (experiment C) were assayed. A single UV dose of 16 J/m^2 was given. Either 0.05 μg of NQO per ml, 0.01% MMS, 0.2% EMS, or 8.5 nM MNNG was added to the growth medium. Haploid yeast strains contained the indicated fusions. The *his3-lacZ* fusion was as either a single integrated copy (*his3a*) or multicopy on a replicating plasmid (*his3b*). Each value is the mean (± standard deviation) specific activity of three cultures. The treated cultures with the *his3-lacZ* fusions were not significantly different ($P \leq 0.01$) from the control cultures except for the MMS-treated cultures in which the means were significantly different ($0.01 \leq P \leq 0.05$). Each value is the mean of three determinations from a single culture.

[b] ND, Not determined.

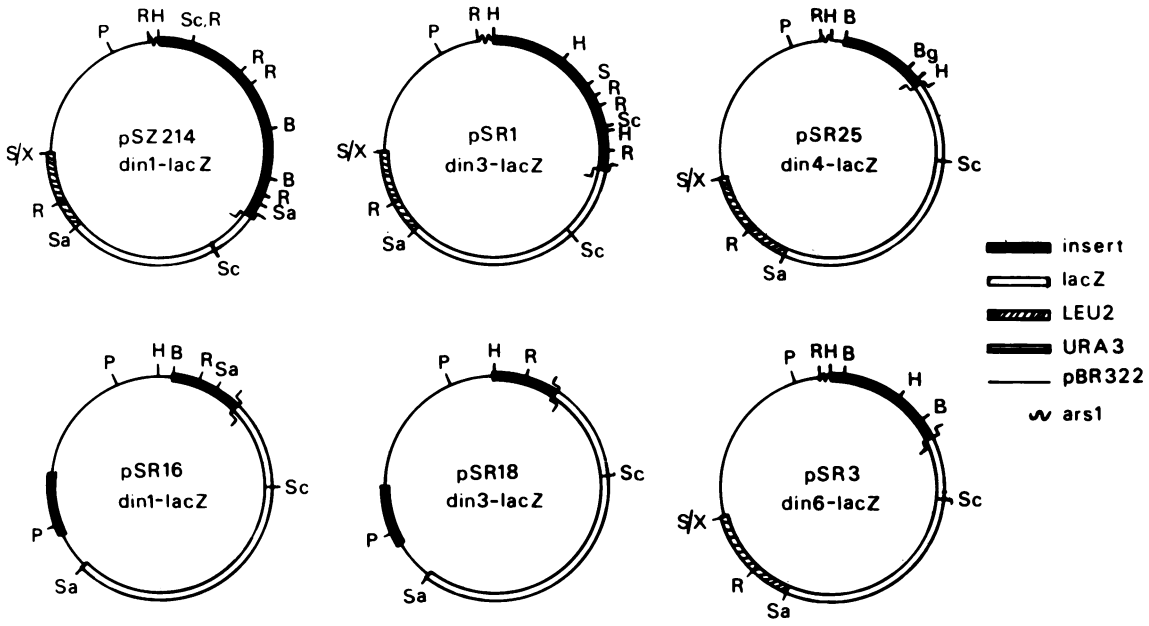

FIG. 2. Restriction endonuclease site maps of the *din-lacZ* fusion plasmids. The restriction endonuclease site maps of the *din* fusions *din1*, *din3*, *din4*, and *din6* are shown. Plasmids pSZ214, pSR1, pSR25, and pSR3 were extracted from *S. cerevisiae* transformants as described in the text. Restriction cleavage sites are indicated: B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sc, *Sac*I; St, *Stu*I; X, *Xho*I. The junction of the pBR322 and *S. cerevisiae LEU2* sequences is indicated (S/X). The approximate positions of the fusion junctions are indicated (S/B). pSR1 lacks *Pst*I and *Bgl*II sites in the *S. cerevisiae* insert. The sites for these two endonucleases in the inserts of pSZ214, pSR3, and pSR10 have not been mapped. pSZ214 and pSR3 lack the 346-base-pair *Bam*HI-*Hin*dIII pBR322 fragment because these plasmids were obtained from *Hin*dIII-cut yeast DNA. An *Xho*I site was not found in pSR1, although it was expected to be in the insert because this enzyme was used to isolate the plasmid. Plasmids pSR16 and pSR18 were constructed as described in the text and were cut with *Eco*RI for integrating the *din1*- and *din3-lacZ* fusions into genomic yeast DNA by transformation.

grown preparatively in *E. coli* and analyzed with restriction endonucleases. The restriction maps of four *din* fusion plasmids (Fig. 2) are clearly different. The *din2* and *din5* fusion plasmids have not yet been fully characterized.

To determine that each *din* fusion was made to a different gene and that the yeast sequences in a fusion had not been altered during the construction of the fusions or isolation of the plasmids, each of the four mapped *din* fusions was used as a probe in DNA blotting experiments. Total *S. cerevisiae* genomic DNA from a wild-type strain lacking any plasmid or *lacZ* sequence was cleaved with a variety of restriction enzymes in single or double digests, electrophoresed on agarose gels, and analyzed by Southern blotting. Each *din* fusion was used in turn as a probe to determine the sizes of homologous fragments of genomic DNA. Fragments consist-

TABLE 3. Expression of integrated *din-lacZ* fusions[a]

Strain	*din-lacZ* fusion	No. of integrated copies	U of beta-galactosidase[b]	
			−MMS	+MMS
T30-15	1	1	0.77 ± 0.04	65 ± 0.7
T30-11	1	1	0.54 ± 0.01	52 ± 1.4
T30-23	1	2–4	0.67 ± 0.12	48 ± 12
T31-27	3	1	0.73 ± 0.17	2.3 ± 0.17
T31-39	3	1	0.12 ± 0.06	2.2 ± 0.01
T31-18	3	2–4	0.19 ± 0.04	6.1 ± 0.08

[a] The cultures were assayed after growing for 5 h in either 0.01% MMS or no MMS.

[b] Each value is the mean (± standard deviation) specific activity of duplicate cultures.

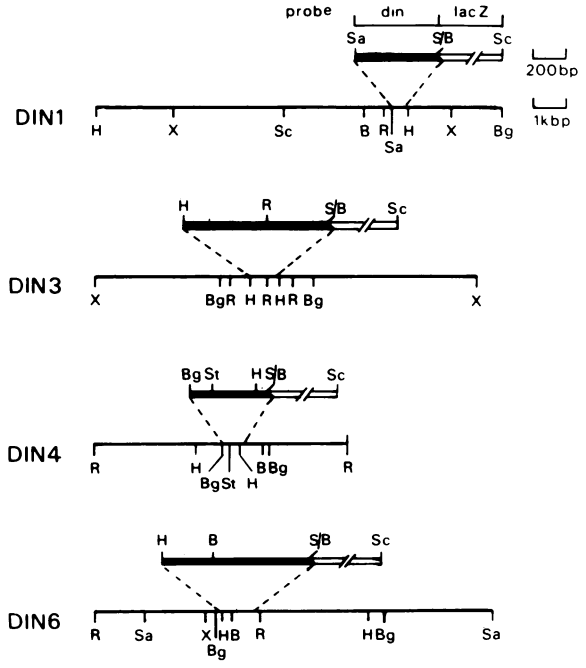

FIG. 3. Restriction endonuclease site maps of the genomic *DIN* genes. The maps were deduced from the sizes of restriction fragments homologous to the *DIN* gene probes shown above each gene. The restriction enzyme cleavage sites are designated as in Fig. 2. Only the sites within or immediately adjacent to the probe could be mapped as explained in the text.

ing of 500 to 1,000 base pairs of yeast insert sequences immediately adjacent to the *lacZ* sequence were used as probes. These sequences (Fig. 3) were subcloned into pBR322 or pMC1403 as described above to purify them away from any unrelated yeast sequences.

The small *din* probes yielded simple, consistent patterns when hybridized to the Southern blots of genomic *S. cerevisiae* DNA (Fig. 4). None of the probes hybridized to any major repetitive sequences. The restriction fragments homologous to each of the probes from the four fusions are different, showing that each corresponds to a unique gene. For example, the *din1*, *din4*, and *din6* probes each hybridized to a single *Eco*RI restriction fragment (of 5.2, 7.6, and 4.7 kb, respectively), whereas the *din3* probe hybridized to two *Eco*RI fragments (0.9 and 1.0 kb) as expected because this probe spans an *Eco*RI site. The patterns of hybridization also show that the sequences in each probe are normally contiguous in genomic DNA.

Data from these and other Southern blots (not shown) were used to generate restriction maps for four of the wild-type, intact *DIN* genes (Fig. 3). No similarities are apparent among any of the maps, again showing that the fusions represent four different genes. As suggested by the finding that there are no *Bam*HI sites at the *lacZ* fusion junctions, there are no *Bam*HI sites on the genomic *DIN* genes at locations corresponding to the junction sites. Because the mapping was done with small probes, sites removed from the region of homology may have gone undetected. For example, although the two *Eco*RI fragments on the *DIN3* map could be placed, because of their homology to the probe and the presence of the *Eco*RI site in the probe sequence, other *Eco*RI fragments within the larger *Xho*I fragment could exist but be undetectable in the *Xho*I-*Eco*RI double digest of genomic DNA.

We limited the size of the probes used in the above mapping experiments because we often found that the yeast

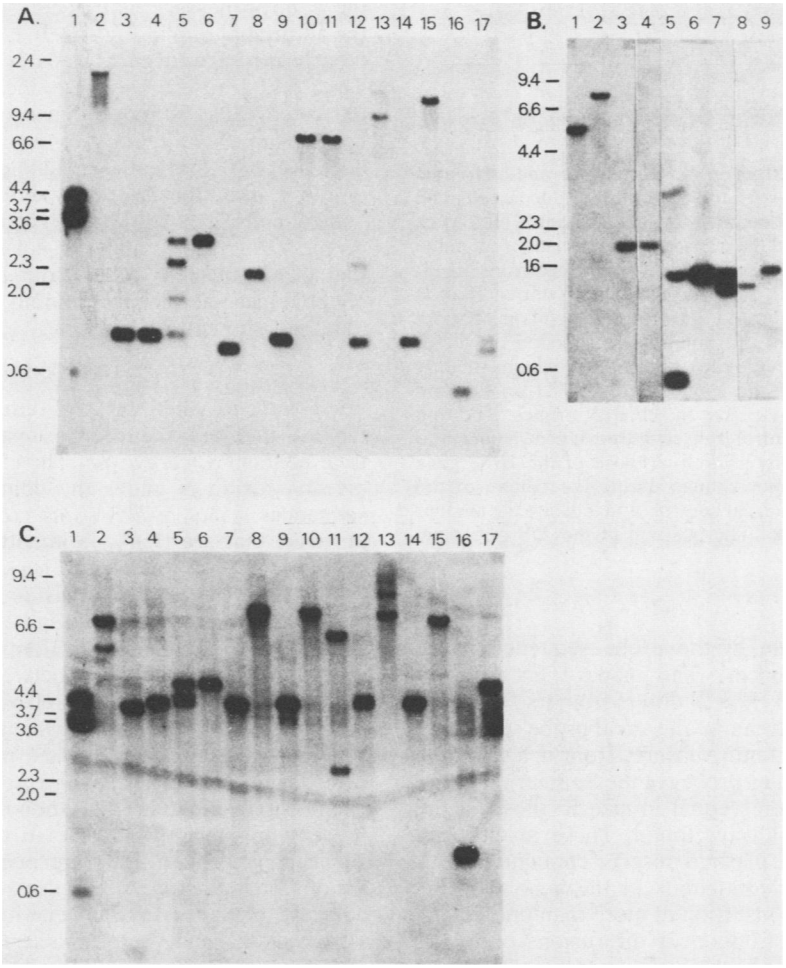

FIG. 4. *S. cerevisiae* genomic DNA restriction fragments homologous to *din* fusions. The three autoradiograms are of Southern blots of *S. cerevisiae* genomic DNA hybridized with probes specific for *din3* (A) *din4* (B), and *din6* (C). These probes, as illustrated in Fig. 3, were from plasmids pSR8, pSR36, and pSR10, respectively. *S. cerevisiae* DNA from strain A15 lacking any vector plasmid or fusion was cleaved with one or two restriction endonucleases, fractionated by size on 0.5% agarose gels, transferred to nitrocellulose paper, and hybridized with the probes. Lanes 1 of A and C have pBR322 DNA cut with *Eco*RI, *Hin*dIII and *Sal*I, or *Nar*I. Lanes 2 to 17 of A and C have A15 DNA cut with the following enzymes (per lane): 2, *Bam*HI; 3, *Bam*HI and *Hin*dIII; 4, *Hin*dIII; 5, *Bgl*II and *Hin*dIII (partial); 6, *Bgl*II; 7, *Pst*I and *Hin*dIII; 8, *Pst*I; 9, *Sal*I and *Hin*dIII; 10, *Sal*I; 11, *Bam*HI and *Sal*I; 12, *Kpn*I and *Hin*dIII; 13, *Kpn*I (partial); 14, *Xho*I and *Hin*dIII; 15, *Xho*I; 16, *Eco*RI and *Hin*dIII; 17, *Eco*RI. Lanes 1 to 9 of B have A15 DNA cut with the following enzymes (per lane): 1, *Bam*HI and *Eco*RI; 2, *Eco*RI; 3, *Eco*RI and *Hin*dIII; 4, *Hin*dIII; 5, *Hin*dIII and *Bgl*II (partial); 6 and 9, *Bgl*II; 7, *Bam*HI and *Bgl*II (partial); and 8, *Bam*HI and *Bgl*II. A partial digestion by an enzyme is indicated in the above list; (partial). The molecular weights in kilobase pairs of the marker DNAs are indicated.

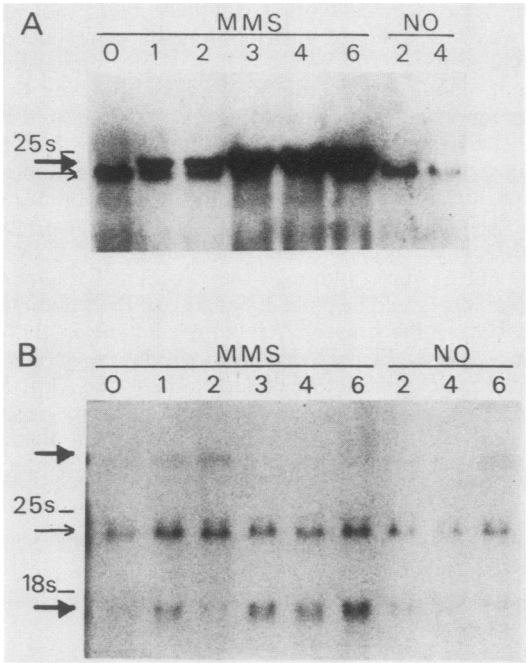

FIG. 5. *DIN* gene transcripts in MMS-induced and untreated cells. Northern blots of RNA from 0.01% MMS-induced cells and untreated cells were hybridized with the ^{32}P-labeled *DIN1* (A) or *DIN3* (B) specific probes, and the transcripts were visualized on the autoradiograms shown here. The RNA was extracted from MMS-treated cells grown for 0, 1, 2, 3, 4, and 6 h or from untreated cells grown for 2, 4, and 6 h as indicated at the top of each lane. The A2 cells used in this experiment did not have any fusion or vector sequence so that the genomic gene transcripts could be readily visualized. The thick arrows designate the 3-kb (A) or 4.4- and 1.8-kb (B) transcripts that hybridize to the *DIN* probes. The thin arrow indicates the 2.8-kb control transcript from a gene adjacent to the *HIS3* gene (51): its levels, as well as those of the *HIS3* gene transcript (not shown), do not change during the course of the experiment and thus act as internal controls and indicate the amount of RNA present in each lane. The position of the 25S ribosomal RNA is shown.

insert sequences upstream of the probe sequences were unrelated genomic sequences. The map of each fusion plasmid originally isolated (Fig. 2) differs from its *DIN* gene (Fig. 3) at some point upstream of the small probe sequence. When large fragments or entire inserts from a few of the fusion plasmids were used as probes in the Southern blotting analyses, genomic fragments equal in size to those in the fusion plasmid were not always found. These results suggested that many of the plasmid inserts contained DNA fragments that were not contiguous in the genome. This could be due either to the insertion of more than one *Sau*3A fragment during the construction of the fusions or to the cloning of multiple, independent restriction fragments during the isolation of the fusion plasmids.

***DIN* transcripts.** We have characterized the transcripts of the *DIN1* and *DIN3* genes and studied the changes in their levels in MMS-induced cells (Fig. 5). To analyze the transcripts of intact genomic *DIN* genes rather than those of *din-lacZ* fusions, we used an *S. cerevisiae* strain that did not contain any fusion or vector sequences as a source of RNA in these experiments. The hybridization probes used to detect *DIN* transcripts in RNA gel blots were the same as those described above in the DNA blotting experiments.

The *DIN1* probe hybridizes to a single 3-kb transcript. Analysis of oligo(dT)-selected mRNA does not reveal any additional transcripts (data not shown). The 3-kb mRNA is detectable as early as 1 h after the addition of MMS and increases in abundance with time, whereas it is undetectable in untreated cells (Fig. 5A). The induction kinetics of the *DIN1* transcript are similar to the induction kinetics of the *din1-lacZ* fusion (Fig. 2 and 5A). The induction in MMS-treated cells is similar to that in NQO-treated cells (42). The levels of *HIS3* mRNA and mRNA from a gene adjacent to *HIS3* do not change with time in either treated or untreated cells.

The same RNA preparations that were used to study the *DIN1* transcript were used to identify the *DIN3* transcript. Two transcripts, of 1.8 and 4.4 kb, hybridized to the 1.1-kb *DIN3* probe (Fig. 5B). The abundance of the 1.8-kb transcript began to increase between 1 and 3 h after MMS addition and remained above control levels at 6 h. The transcript shows a fivefold increase from 0 to 6 h relative to the control message as detected by densitometric scans of the autoradiogram in Fig. 5B (0.2, 0.3, 0.9, and 1.1 times the control message levels at 0, 1, 3, 4, and 6 h, respectively). The 1.8-kb transcript seems to increase temporarily at 2 h in untreated cells, although densitrometic scans show that it has the same level relative to the control message at 2 h as it does at 0 h (0.2 times). No significant change in the amount of the 4.4-kb transcript was detected in either treated or control cells. The 1.8-kb transcript is most likely a product of the *DIN3* gene, and the 4.4-kb transcript is probably that of an adjacent gene. Both transcripts are present in oligo(dT)-selected mRNA and no other transcripts are seen (data not shown).

DISCUSSION

We have isolated six different *S. cerevisiae* gene *lacZ* fusions that are induced in response to DNA-damaging treatments in *S. cerevisiae*. The expression of the *lacZ* gene in each fusion is under the control of the 5′ yeast gene sequences. Thus, *S. cerevisiae* probably contains a large set of genes that are transcriptionally activated as part of the cellular response to DNA damage.

Our analysis of *S. cerevisiae DIN* genes parallels the earlier analysis by Kenyon and Walker (20) of *E. coli din* genes. They used the Mu *d*1(Ap^r *lac*) fusion vector (8) to generate a library of fusions between *E. coli* and *lacZ* genes, from which they isolated a set of damage-induced fusions. These bacterial fusions differ from our yeast gene fusions in several respects. The Mu *d*1 phage creates fusions by transposing into genes on the *E. coli* chromosome. Thus, each fusion generates a mutation in the bacterial gene and is present on the chromosome in the original location as a single copy. The fusions are operon (i.e., transcriptional) fusions, so that expression reflects promoter activity only. In contrast, our *S. cerevisiae* gene fusions were constructed in vitro on a plasmid vector and screened after transformation into yeast cells. This method of generating fusions does not mutate the original chromosomal gene, so that autoregulated genes and genes with essential functions can be identified. The fusions can be either chromosomal and low copy or episomal and high copy. They are protein fusions and thus produce hybrid beta-galactosidase proteins.

It seems likely that all six of our fusions are joined to transcriptionally induced chromosomal *DIN* genes. We examined the mRNAs transcribed from the unaltered chromosomal *DIN1* and *DIN3* genes. In both cases, the mRNA levels increased after DNA-damaging treatments. Many

other yeast gene *lacZ* fusions also have been shown to be regulated in the same way as their intact chromosomal genes (11, 15, 30, 36, 41, 47).

The *din-lacZ* fusions are not all coordinately regulated. Five of the six fusions are induced by most of the DNA-damaging treatments that we tested. The *din5-lacZ* fusion, however, is induced by UV and thymine starvation but not by the alkylating agents MMS, EMS, or MNNG. *S. cerevisiae* is known to have at least three major pathways for the repair of DNA damage. It is possible that distinctly regulated *DIN* genes function in the repair of specific DNA lesions. *S. cerevisiae*, like *E. coli*, may have separately regulated groups of genes involved in responses to different forms of DNA damage.

The *din1*- and *din3-lacZ* fusions have low basal levels of expression and high induction ratios, and the original isolates were mitotically stable. The subcloned fusions behave similarly when reintroduced into yeast cells by integration at the homologous genomic site. The remaining four fusions were originally mitotically unstable, and all had high basal levels of expression and low induction ratios. The fact that a mitotically stable derivative of the *din5-lacZ* fusion shows a low basal level and high induction ratio suggests that the presence of the *din2*, *din4*, *din5*, and *din6* fusions on autonomous plasmids may affect their expression or regulation and be responsible for the high basal levels and low induction ratios. However, this possibility has not yet been directly tested by the reintroduction of the fusions into *S. cerevisiae* on integrating plasmids.

Since the *din-lacZ* fusions were created in a manner that did not mutate the chromosomal *DIN* genes, we have no indications of the functions of any of these genes. The *S. cerevisiae DIN* genes could be active in repair, recombination, mutagenesis, or general stress recovery. Nearly 100 loci involved in repair or recombination in yeasts have been identified genetically, and many of these have recently been cloned. The restriction maps of the *DIN1*, *DIN3*, *DIN4*, and *DIN6* genes are different from those of the *RAD3* (18, 32), *RAD1* (19), *RAD2* (33), and RAD10 (Weiss and Friedberg, personal communication), *RAD6* (38), *RAD50* (22), *RAD52* (44), and *RAD51*, *RAD54*, and *RAD55* (7) genes.

Several DNA damage-inducible yeast genes have recently been found by others by different techniques. McClanahan and McEntee (personal communication) screened a lambda library of yeast genomic sequences by differential plaque hybridization and isolated 10 clones representing at least six DNA damage-inducible genes. Barker (personal communication) has recently found that the *CDC9* gene, which encodes DNA ligase, is induced in response to DNA damage. The restriction maps of the *DIN1*, *DIN3*, *DIN4*, and *DIN6* genes are different from those of the *DIN* genes cloned by McClanahan and McEntee, and from that of *CDC9* (4).

How many *S. cerevisiae* genes are *DIN* genes? Four of the *DIN* genes that we identified came from a library of transformants that contained fusions to ca. 500 *S. cerevisiae* genes. Therefore, assuming that *S. cerevisiae* have ca. 10,000 genes, roughly 80 should be DNA damage inducible. This is consistent with the fact that none of our six *DIN* genes are the same as the *DIN* genes of McHanahan and McEntee, or are *CDC9*. In addition, some DNA repair genes are constitutively expressed (19, 32). Thus the total number of genes involved in the response to DNA damage may be significantly greater than the number of *DIN* genes. It appears that yeast cells, like *E. coli*, have devoted ca. 1% of their genetic capacity to the response to DNA damage. The existence of DNA damage-inducible genes in yeast cells, a eucaryote, and in *E. coli* and other procaryotes (52) suggests that such genes may be ubiquitous. Experiments on the inducibility of particular functions in mammalian cells imply the existence of *DIN* genes in mammals (52).

The cloned *DIN* genes and *din-lacZ* fusions will have several applications in the study of the DNA damage response. Sequencing and deletion analysis should reveal *cis*-acting control sequences. The fusions should be particularly useful in the isolation of mutants defective in *trans*-acting regulatory signals. The *din-lacZ* fusions also may have a practical application in a short-term assay for the detection of genotoxic and carcinogenic compounds. Most genotoxic agents induce the SOS response in *E. coli*, and the *sfiA::lacZ* fusion has been used in a screening assay (39). The *S. cerevisiae din* fusions could provide a similar assay in a eucaryotic host.

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