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DNA Damage Induction of Ribonucleotide Reductase

STEPHEN J. ELLEDGE[1]* AND RONALD W. DAVIS[2]

Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030,[1] and Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305[2]

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RNR2 **encodes the small subunit of ribonucleotide reductase, the enzyme that catalyzes the first step in the pathway for the production of deoxyribonucleotides needed for DNA synthesis.** ***RNR2*** **is a member of a group of genes whose activities are cell cycle regulated and that are transcriptionally induced in response to the stress of DNA damage. An** ***RNR2-lacZ*** **fusion was used to further characterize the regulation of** ***RNR2*** **and the pathway responsible for its response to DNA damage. β-Galactosidase activity in yeast strains containing the** ***RNR2-lacZ*** **fusion was inducible in response to DNA-damaging agents (UV light, 4-nitroquinoline-1-oxide [4-NQO], and methyl methanesulfonate [MMS]) and agents that block DNA replication (hydroxyurea [HU] and methotrexate) but not heat shock. When** ***MAT*****a cells were arrested in G1 by α-factor,** ***RNR2*** **mRNA was still inducible by DNA damage, indicating that the observed induction can occur outside of S phase. In addition,** ***RNR2*** **induction was not blocked by the presence of cycloheximide and is therefore likely to be independent of protein synthesis. A mutation,** ***rnr2-314****, was found to confer hypersensitivity to HU and increased sensitivity to MMS. In** ***rnr2-314*** **mutant strains, the DNA damage stress response was found to be partially constitutive as well as hypersensitive to induction by HU but not MMS. The induction properties of** ***RNR2*** **were examined in a** ***rad4-2*** **mutant background; in this genetic background,** ***RNR2*** **was hypersensitive to induction by 4-NQO but not MMS. Induction of the** ***RNR2-lacZ*** **fusion in a RAD$^+$ strain in response to 4-NQO was not enhanced by the presence of an equal number of** ***rad4-2*** **cells that lacked the fusion, implying that the DNA damage stress response is cell autonomous.**

The capacity to efficiently sense and respond to environmental stress is central to the ability of an organism to undergo complex developmental transformations and to successfully adapt to changing environmental conditions. Two fundamental types of sensory networks appear to be ubiquitous among organisms: the ability to recognize and respond to thermal shock (27) and the ability to recognize and respond to DNA damage (2, 21, 32, 40, 41, 46). In addition, several genes that are transcriptionally activated in response to both types of stress have been identified (24, 33), suggesting a potential interaction between both sensory networks.

The regulatory circuitry controlling the response to thermal shock has been extensively studied in both procaryotes and eucaryotes, and the transcription factors that mediate this response have been identified (13, 43, 49). The response to DNA damage has also been extensively studied but, in contrast, is well understood only in the procaryote *Escherichia coli*. Treatment of *E. coli* with agents that damage DNA or block replication causes the appearance of a set of physiological responses, including the induction of DNA repair processes, mutagenesis, and induction of lysogenic bacteriophage (for a review, see reference 47). These processes have collectively been called the SOS response because at least some of them appear to promote cell survival. In all, over 20 genes that are transcriptionally activated in response to DNA damage have been identified. The molecular mechanism of this coordinately regulated response involves the proteolytic inactivation of a common repressor, the LexA protein, by an activated form of the RecA protein. The functions of several SOS-regulated genes are known and include excision repair (*uvrAB*) (22), recombinational repair (*recA*) (34), SOS repression (*lexA*) (3, 28), mutagenesis (*umuCD* and *mucAB*) (10, 11), inhibition of cell septation (*sfiA*) (16), and possibly site-specific recombination (*himA*) (35, 37).

Less is known about the stress response to DNA damage in eucaryotes. In *Saccharomyces cerevisiae*, a number of genes have been identified on the basis of increased transcription in response to DNA damage: the *DIN* (40) and *DDR* (32) genes. However, the functions of these genes remain unknown. Several genes of known function also have been shown to be inducible by DNA damage; these include *RAD2* (excision repair) (39), *RAD54* (recombinational repair) (7), *UBI4* (protein degradation) (44), *CDC9* (DNA ligase) (38, 48), and *POL1* (DNA polymerase 1) (20). We have previously shown that the transcripts for *CDC8* (thymidylate kinase) and *RNR2* (ribonucleotide reductase) are inducible in response to DNA damage (9). These genes may be members of a global regulon of genes with cell cycle-regulated activities involved in DNA replication (including *CDC9* and *POL1*) that have evolved a mechanism allowing additional cell cycle-independent expression in response to the stress of DNA damage.

Virtually nothing is known about the mechanisms that sense and respond to DNA damage in eucaryotes. Although several inducible genes have been identified, it has yet to be demonstrated that the inducibility of any one gene is important physiologically in the cellular response to damage. To determine the role that the induction processes play in the cellular response to damage, mutations in the sensory pathway(s) are needed. The first step toward understanding the complex mechanisms involved in this eucaryotic sensory pathway(s) is to carefully define and characterize the cellular response to DNA damage. We have therefore undertaken a detailed analysis of the response of the *RNR2* gene to DNA damage. We have examined the range of agents that elicit the response, the cell cycle stage dependence of the response, and the effects of various mutations on the response.

* Corresponding author.

TABLE 1. Strains used

Strain	Genotype
LN114	*MATa ura3-52 Δhis3-200 trp1-289 ade2-101*
SX46	*MATa ura3-52 his3-832 trp1-289 ade2-101*
SX46 *rad4*	*MATa ura3-52 his3-832 trp1-289 ade2-101 rad4-2*
RC634	*MATa sst1-3 rme1 ade2 his6 met1 ura1 can1 cyh2*
YNN345	*MATa ura3-52 Δhis3-200 Δtrp1-901 leu2-3,112*
YNN346	LN114 pNN403 (*URA3 RNR2-lacZ*)
YNN347	*MATα ura3-52 Δhis3-200 lys2-801 ade2-1 Δtrp1-901*
YNN348	*MATa ura3-52 Δhis3-200 lys2-801 ade2-1 Δtrp1-901 rnr2-314(TRP1)*
YNN349	YNN347 pNN405 (*URA3*)
YNN350	YNN348 pNN405 (*URA3*)
YNN351	YNN346 pRS46 (*TRP1*)
YNN352	YNN346 pNN404 (*TRP1 RNR2*)
YNN353	SX46 pNN405 (*URA3 RNR2-lacZ*)
YNN354	SX46 *rad4-2* pNN420 (*URA3 RNR2-lacZ*)

MATERIALS AND METHODS

Media and chemicals. Yeast minimal medium contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) and 2% glucose; 2% agar (Difco) was added for solid media. The selective medium used was minimal medium supplemented with various amino acids and bases, prepared as described by Sherman et al. (42), as was yeast extract-peptone-dextrose medium (YPD). Methyl methanesulfonate (MMS) was purchased from Eastman Kodak Co. (Rochester, N.Y.), and yeast α-factor, 4-nitroquinoline-1-oxide (4-NQO), and hydroxyurea (HU) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Strains and plasmids. The yeast strains used are given in Table 1. *E. coli* JM107 was used as a host for constructions and plasmid amplification. The plasmid used as a base for pNN405, pLG312 ΔSS (9, 15), was a gift of A. Mitchell. pLG312 ΔSS is a derivative of pLG312 with a *Bgl*II linker inserted between the *Sal*I and *Sma*I sites, thus removing the upstream activation sequence of *CYC1*. pRS46 is a 2μm-based *TRP1* vector provided by R. Sikorfki and J. Shero. pC4-β-gal was a gift of C. Thummel (C. S. Thummel, A. M. Boulet, and H. Lipshitz, Gene, in press). Other plasmids used in this study are described below.

Construction of *RNR2-lacZ* fusions. *RNR2-lacZ* fusions were constructed in several steps. First, the *Pvu*II-*Nsi*I fragment of *RNR2* was cloned into *Sma*I-*Pst*I-cut pIC19R (31) to form pSE551. This fuses the coding region of *RNR2* at the *Nsi*I site to a polylinker with the following sites after the *Nsi*I-*Pst*I hybrid site: *Hin*dIII *Nru*I *Sac*I *Xho*I *Bgl*II. The *Bgl*II site places the *RNR2* gene in the proper reading frame to be fused to the *Bam*HI site of the 5′-truncated *lacZ* fragment in pC4 (6; Thummel et al., in press) to produce a protein fusion. This construct also places an *Eco*RI site near the *Sma*I-*Pvu*II hybrid site. The *Eco*RI-*Bgl*II fragment of pSE551 was cloned into *Eco*RI-*Bam*HI-cut pC4-β-gal. The entire *RNR2-lacZ* fusion can be excised from this construct on an *Eco*RI-*Xba*I fragment. The *Eco*RI-*Xba*I fragment, with the *Xba*I site filled with the Klenow fragment of DNA polymerase I, was cloned into *Eco*RI-*Sma*I-cut pSE273, a *URA3 ARS1 CEN4* vector (S. J. Elledge and R. W. Davis, unpublished data) to produce pNN403. The map is shown in Fig. 1.

pNN405 was constructed by first cloning the *Hin*dIII-*Xho*II fragment (made flush with the Klenow fragment of DNA polymerase I) into *Hin*dIII-*Hin*dII-cleaved pBS KS+ (Stratagene, San Diego, Calif.). The upstream region of *RNR2* was excised from this plasmid on a *Bam*HI-*Xho*I fragment and was cloned into pLG312 ΔSS in a three-way ligation involving the *Bgl*II-*Sac*I fragment of pLG312 ΔSS containing the 5′ end of the *CYC1-lacZ* fusion and the *Xho*I-*Sac*I fragment containing the 3′ end of the *lac*Z gene and the remaining plasmid sequences. This construct, pNN405, contains the *RNR2* regulatory region in front of the *CYC1* TATA box. The *RNR2* sequences are in the opposite orientation relative to the *CYC1* TATA in pNN405 as they are relative to the TATA in the native *RNR2* promoter. The validity of this construct was verified by introduction into LN114 via transformation and showing induction of β-galactosidase activity in response to treatment with HU.

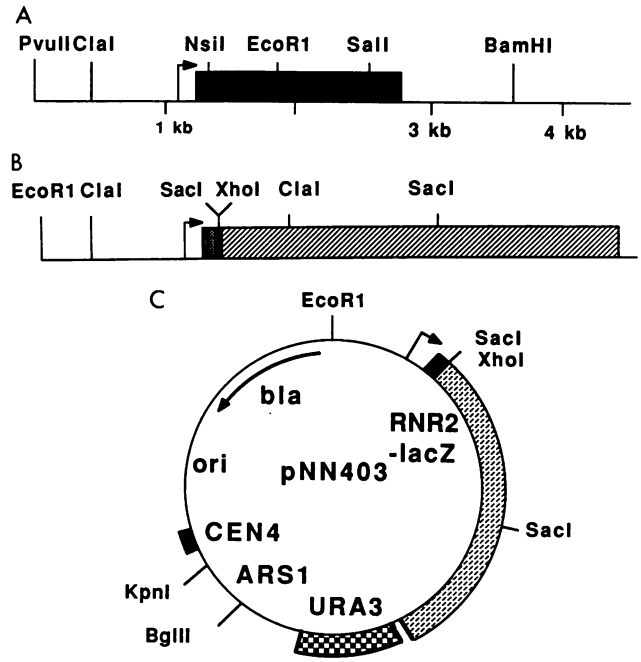

FIG. 1. Schematic representation of the *RNR2* and *RNR2-lacZ* fusion genes. (A) Restriction map of the DNA encoding the *RNR2* locus on chromosome X. Symbols: ▨, the *RNR2* structural gene; →, initiation of transcription of *RNR2*. kb, Kilobases. (B) Restriction map of the *RNR2-lacZ* fusion created at the *Nsi*I site (see Materials and Methods). Symbols: ▨, amino terminus of *RNR2*; ■, polylinker sequence from pIC19R used to adjust the *RNR2* reading frame; ▨, the *lacZ* gene deleted for the first eight amino acids (6). (C) Restriction map of pNN403, the yeast centromeric plasmid containing the *RNR2-lacZ* fusion used to assay for DNA damage induction.

Effects of overproduction of *RNR2*. We have previously shown that overproduction of the small subunit of ribonucleotide reductase can be accomplished by placing *RNR2* on the 2μm vector YEp24 (9). pNN404 was constructed by cloning the *Bam*HI fragment of pNN315 (9) containing *RNR2* into *Bam*HI-cleaved pRS46, a 2μm-based *TRP1* vector. pRS46 and pNN404 were each placed into strain YNN346 (already containing pNN403) to create YNN351 and YNN352, respectively. These strains were tested for the ability to induce β-galactosidase activity in response to MMS and HU treatment.

Assay for β-galactosidase activity. β-Galactosidase assays of *S. cerevisiae* with the colorimetric substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were done as described by Guarente (14). Yeast strains were grown overnight in selective minimal medium for all experiments described in this paper. They were diluted into fresh YPD and grown to mid-log phase (optical density at 600 nm [OD_{600}] of 0.3), and

the various inducing agents were added directly to the liquid medium (with the exception of UV, which is discussed below). After treatment, 3 ml of cells was pelleted, washed once with water, and suspended in 1.0 ml of sterile water. A 0.5-ml sample of cell suspension was added to 0.5 ml of 0.37% formaldehyde solution to fix the cells for later determination of OD_{600}. The remaining 0.5 ml was added to 0.5 ml of Z buffer in a glass test tube, to which 50 μl of $CHCl_3$ and 25 μl of 0.1% sodium dodecyl sulfate were then added. The samples were vortexed in groups of four for exactly 1 min and then incubated at 28°C for 10 min before the reaction was started by addition of 0.2 ml of a 4-mg/ml concentration of ONPG. The rest of the assay and unit calculations are as described by Miller (36). This regimen produced activity measurements that were reproducible within 20%.

All other assays for β-galactosidase (those shown in Fig. 6 to 9) were performed by adding the inducing agent at the indicated concentrations directly to the liquid medium in which the cells were growing and incubating the cultures for 4 h except for the assay shown in Fig. 2; this was a time course, and samples were taken at the indicated times.

UV and heat shock assays. In general, DNA-damaging agents were added directly to early- to mid-log-phase cells (OD_{600} of 0.1 to 0.3) growing in liquid culture (YPD) at 30°C and left in for the duration of the assay. Experiments involving the exposure of cells to UV light were performed by growing cells in minimal medium, exposing them to a UV fluence of 1 J/m^2 per s while shaking the cells in a glass petri dish to minimize the effects of shielding. After irradiation, cells were grown at 30°C in the dark to avoid photoreactivation. For the heat shock experiment, cells were grown at 23°C in YPD and then resuspended in YPD at 37°C and incubated for 2 h before assays for induction. Control samples were treated similarly to experimental samples except for UV or heat shock.

Cell cycle and HU and MMS induction. Strain RC634 was examined to determine whether the stage of the cell cycle affects induction of *RNR2*. RC634 contains the *sst1* mutation, which eliminates a protease that normally degrades α-factor. Consequently, this strain is much more sensitive to the presence of α-factor. Yeast α-factor causes the arrest of *MAT***a** cells at the G1 stage of the cell cycle (4). We grew RC634 in YPD to an OD_{600} of 0.3 and then added α-factor directly to the medium at a concentration of 10 μM. After 2.5 h, these cells were monitored for schmooing, the characteristic elongated morphology which indicates G1 arrest by α-factor. More than 99% of the cells exhibited such morphology at this point. Then either HU was added to a final concentration of 100 mM or MMS was added to a final concentration of 0.01% (vol/vol), and cells were incubated for an additional 4 h. Cells that were not exposed to α-factor were also treated with HU or MMS as cycling controls. Cells treated with α-factor but not HU or MMS were monitored after 4 h for the appearance of buds; 99% of the cells remained unbudded, although they maintained a rather exaggerated elongated morphology. After 4 h, cells were harvested and RNA was prepared for Northern (RNA) analysis as described previously (9). The probe for the Northern blot was the internal *Hin*dIII fragment of *RNR2*. All probe labeling and hybridizations were carried out as described previously (9).

Determination of the requirement for protein synthesis. To determine whether the induction of *RNR2* by 4-NQO was dependent on protein synthesis, we grew YNN346 to mid-log phase in YPD. To half of this culture, cycloheximide (Sigma) was added to a final concentration of 100 μg/ml and incubated at 30°C for 15 min. Then to portions of the samples with and without cycloheximide, 4-NQO was added at 0, 0.25, 0.5, and 1.0 μg/ml, and samples were incubated at 30°C for 4 h. After 4 h, samples were split for RNA preparation or β-galactosidase determination. The probe used for the RNA blot was the same internal *Hin*dIII fragment described above. This probe will hybridize only to the native *RNR2* message because the *RNR2-lacZ* message does not contain sequences homologous to the probe. (The RNA for the 0.5-μg/ml point was spoiled and is not shown.) Equal amounts of RNA were loaded per lane and were shown to contain equivalent amounts of *URA3* message in a separate experiment (data not shown).

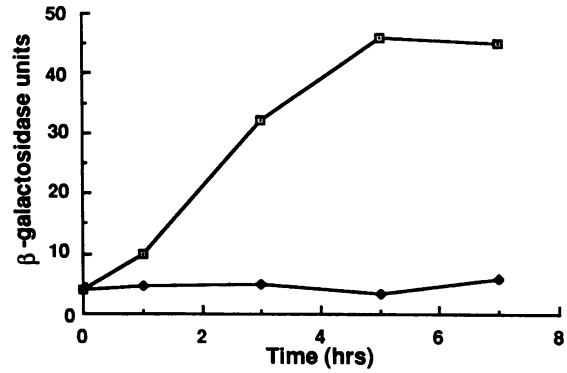

FIG. 2. Induction kinetics of the *RNR2-lacZ* fusion gene treated with 4-NQO. At 0 h, 4-NQO was added to a logarithmically growing culture of YNN346 to a final concentration of 1 μg/ml (□) or cells were untreated as a control (◆). Samples taken at various times were assayed for cell density and β-galactosidase activity as described in Materials and Methods.

Measurements of sensitivity to HU and MMS. The yeast strains YNN347 (RNR^+) and YNN348 (*rnr2-314*) were grown to mid-log phase (10^7 cells per ml), and dilutions of 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} were plated on each of the following plates: YPD or YPD plates supplemented with 1, 5, 20, or 100 mM HU and YPD plates supplemented with 0.0004, 0.002, 0.01, 0.04, or 0.05% MMS. Colony growth was measured after 3 days for HU and after 5 days for MMS.

RESULTS

Construction of an *RNR2-lacZ* fusion. To facilitate the analysis of *RNR2* regulation, a fusion between the *RNR2* and the *lacZ* gene was constructed. This construction, which resulted in the production of a fusion protein with the amino-terminal 12 amino acids of the *RNR2* protein fused to β-galactosidase, is described in Materials and Methods and shown in Fig. 1. The *RNR2-lacZ* fusion on a low-copy-number (*ARS1 CEN4 URA3*) yeast vector was designated pNN403 and was introduced into yeast strain LN114 by transformation, creating strain YNN346. To confirm that this construct directed β-galactosidase synthesis in a manner representative of *RNR2* regulation, YNN346 was treated with 1 μg of 4-NQO per ml, and β-galactosidase specific activity was measured at various time points (Fig. 2). β-Galactosidase activity was induced approximately 11-fold over the levels present in untreated cells, which is less than the 18-fold increase previously observed for the *RNR2* mRNA levels (9). Reduced induction of the fusion may be a result of an increased basal level of transcription on the plasmid due to the influence of vector sequences. Induction of the fusion by 4-NQO was maximal at a concentration of 1

TABLE 2. Induction of the *RNR2-lacZ* fusion on pNN403 by various agents[a]

Agent	Dose	Induction ratio
None		1.0
UV radiation	50 J/m^2	3.5
Methotrexate	10 μg/ml	8.4
4-NQO	1 μg/ml	11
MMS	0.01% (vol/vol)	10.6
HU	100 mM	13.1
Heat shock	23–37°C	1.0
Cycloheximide	100 μg/ml	1.1[b]

[a] Assays were performed after 4 h of treatment of YNN346 with each agent.
[b] Also measured by comparing RNA levels and found to be approximately the same.

μg/ml. A 10-fold increase in the concentration of 4-NQO caused much smaller induction of β-galactosidase activity (data not shown), presumably as a result of excessive cell death. From these data, it can be concluded that the *RNR2-lacZ* fusion on pNN403 is regulated in a manner consistent with that observed for the chromosomal *RNR2* transcript.

***RNR2* is induced by a variety of agents that damage DNA or block DNA replication.** The specificity of the response to DNA-damaging agents can provide information on the nature of the sensory mechanism(s) used by the DNA damage inducibility pathway(s). With this in mind, a variety of agents were tested for their effects on induction of *RNR2-lacZ* expression in YNN346 (Table 2; only doses producing optimal induction are shown). All of the agents that damage DNA (4-NQO, MMS, and UV light) or block DNA replication (methotrexate and HU) caused the induction of *RNR2*. In separate experiments, the chromosomal transcript for *RNR2* has been shown to be inducible by each of these agents (data not shown). However, blocking protein synthesis with cycloheximide or inducing the heat shock response had no effect on *RNR2-lacZ* expression. In the case of cycloheximide, the message levels were also measured and shown to remain uninduced (see below). 4-NQO and the methylating agent MMS gave high levels of induction, in contrast to UV light, which gave only a threefold increase in the level of expression. This effect was not due to the use of a suboptimal UV dose because it was also observed at other doses of UV (data not shown). A more likely explanation is that the other agents were present continually throughout the incubation period, whereas UV treatment occurred only once, at the beginning of the assay period. The UV-induced DNA damage could be repaired, presumably removing the inducing signal, whereas the other agents could continue to damage the DNA, thus maintaining the induction signal.

The most dramatic effect on induction was seen with the antitumor agent HU. HU specifically inhibits ribonucleotide reductase activity by interacting with the small subunit at its unique tyrosyl free radical (1). This inhibition causes the rapid cessation of DNA synthesis. The fact that yeast cells induce *RNR2* expression in response to HU suggests that they have an autoregulatory circuit that maintains the levels of ribonucleotide reductase in vivo. Since ribonucleotide reductase is composed of two nonidentical subunits, any increase in activity upon induction depends on which subunit is rate limiting and whether the second subunit is coordinately regulated. Although the answer to the question of which subunit is limiting is not known, we have recently cloned the yeast genes encoding the large subunit (S. J. Elledge and R. W. Davis, manuscript in preparation) and observed that their transcription is also inducible by DNA damage. Therefore, it is likely that the amount of active enzyme is also increased. These observations suggest that a phenotype potentially associated with mutations that prevent derepression of these genes will be hypersensitivity to HU.

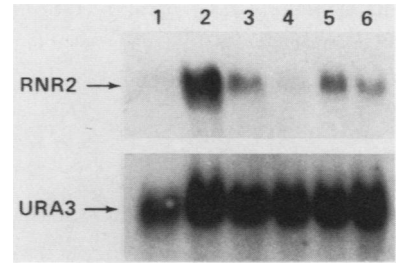

FIG. 3. Induction of the *RNR2* mRNA by HU and MMS in growing cells and during G1 arrest by α-factor. RNA was extracted from untreated RC634 cells (lane 1), 100 mM HU-treated cells (lane 2), and 0.01% MMS-treated cells (lane 3) and from α-factor-arrested RC634 cells with no damage treatment (lane 4), arrested cells treated with 100 mM HU (lane 5), and arrested cells treated with 0.01% MMS (lane 6). Northern blots of these RNAs were hybridized with ^{32}P-labeled *RNR2*- and *URA3*-specific probes, and the transcripts were visualized by autoradiography.

Induction of *RNR2* is independent of the cell cycle. One explanation for why *RNR2* and other genes whose activities are cell cycle regulated respond to DNA damage is that they are needed outside of S phase to produce a metabolic state that facilitates DNA replication needed for repair processes. This hypothesis predicts that *RNR2* induction will occur throughout the cell cycle. To test this hypothesis, RNA was prepared from a yeast strain that was arrested in the G1 phase of the cell cycle with the peptide hormone α-factor (4) and then treated with MMS or HU as described in Materials and Methods while maintaining the presence of α-factor (Fig. 3). The message levels for *RNR2* appeared to increase relative to *URA3* message levels in response to each inducing agent in both cycling and G1-arrested cells. Densitometer tracing of lighter exposures of the *URA3* panel revealed that lane 1 was underloaded by a factor of 2.3 relative to lane 4. The *RNR2* message levels in lanes 1 and 4 were equivalent after normalization to the *URA3* mRNA levels. Interestingly, induction levels for *RNR2* message by HU were about fivefold lower in α-factor-arrested cells than in the cycling population, but α-factor reduced the induction levels for MMS only about twofold.

If HU generates a damage signal by stalling DNA replication through a depletion of deoxynucleoside triphosphates (dNTPs), then it would be surprising to see induction in the absence of S-phase-dependent DNA replication. However, mitochondrial DNA replication proceeds in a cell cycle-independent manner and may be responsible for the generation of a stress signal if nucleotide levels are not maintained in the presence of HU. It is also possible that nucleotide pools turn over at a significant rate in the absence of DNA replication. The fact that induction for HU was lower in G1-arrested cells suggests that an S-phase-dependent function, perhaps chromosomal DNA replication, may contribute to the stress signal produced by HU. Alternatively, there may be an additional contribution to *RNR2* transcription by HU and MMS induction in cycling cells because these agents can temporarily arrest cells in the S phase of the cell cycle. If *RNR2* transcription has a cell cycle component, this induced S-phase arrest can cause an increase in transcription in cycling cells in addition to the increase due to DNA

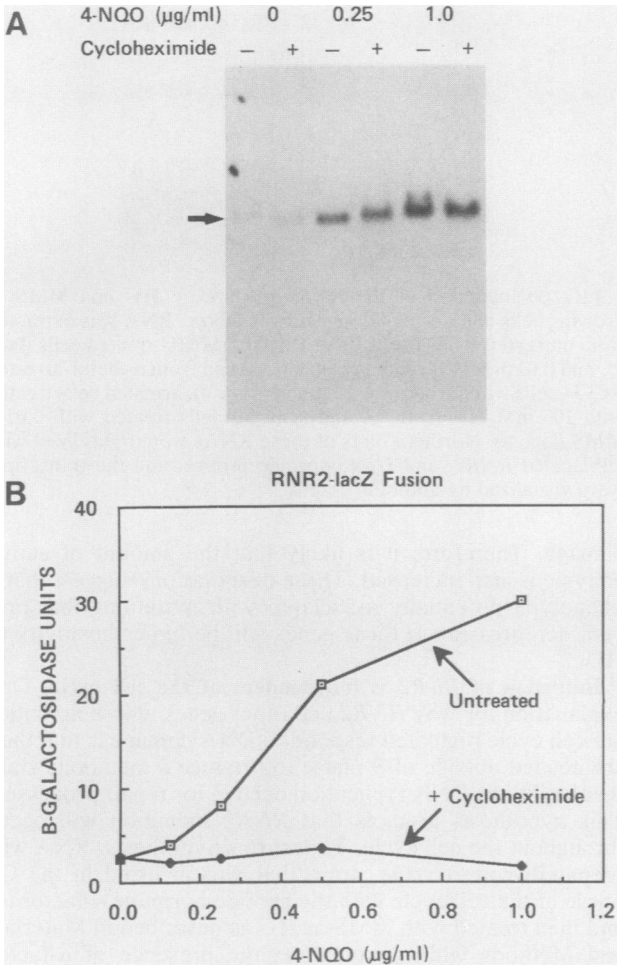

FIG. 4. Demonstration that induction of the *RNR2* transcript by DNA damage is independent of protein synthesis. (A) Northern analysis of RNA extracted from YNN346 cells treated for 4 h with various doses of 4-NQO in the presence (+) or absence (−) of 100 μg of cycloheximide per ml. Blots were hybridized with a ^{32}P-labeled *RNR2*-specific probe that recognizes the native *RNR2* transcript but not the *RNR2-lacZ* transcript. The transcripts were visualized on the autoradiograms shown. (B) β-Galactosidase activities determined for the same cultures and plotted as a function of dose of 4-NQO. One more dose of 4-NQO was used here than in panel A: 0.5 μg/ml. Symbols: ◆, activities measured for cultures treated with cycloheximide; □, control cultures.

damage itself, but this additional contribution is prevented by prior arrest in G1. Clearly, however, these data shown that DNA damage induction can occur outside of S phase.

DNA damage induction of *RNR2* is independent of protein synthesis. A wide variety of mechanisms are used for the signal-induced activation of gene expression in eucaryotic cells. These can be divided into two general categories on the basis of whether or not the biochemical transduction mechanism requires de novo protein synthesis. Treatment with cycloheximide alone does not induce *RNR2* transcription. This fact allowed the determination of whether other agents that do cause transcriptional activation of *RNR2* are dependent on protein synthesis. The presence of cycloheximide did not prevent the induction of *RNR2* mRNA by 4-NQO treatment (Fig. 4A) in YNN346 cells. However, cycloheximide did block induction of the *RNR2-lacZ* fusion protein in response to 4-NQO (Fig. 4B). These data demonstrate that the sensory pathway used for DNA damage induction of *RNR2* is independent of cycloheximide-sensitive protein synthesis, suggesting that the response is mediated by existing cellular factors.

A mutation in *RNR2* confers hypersensitivity to HU. In comparison with other organisms, yeasts are extremely resistant to HU (25). Concentrations of greater than 100 mM are required to prevent growth on plates. Presumably, mutations in the genes encoding ribonucleotide reductase should be able to alter this resistance if HU is acting via inhibition of the enzyme. A mutation in *RNR2*, *rnr2-314*, was described previously (9). In the *rnr2-314* mutant, the carboxy terminus is disrupted by a transplacon insertion, as demonstrated by the loss of the carboxy-terminal epitopes. This mutant strain is viable and grows normally but has an increased propensity to produce the petite phenotype, which is often associated with defects in nucleotide metabolism. *rnr2-314* was tested for sensitivity to HU (Fig. 5A). The *rnr2-314* allele in YNN348 conferred an HU hypersensitivity phenotype on strains relative to the *RNR2* strain YNN347 (YNN347 and YNN348 are sister spore clones). It should be noted that the predominant effect of HU was to severely reduce the growth rate of cells as opposed to killing a certain percentage. In these experiments, an HU concentration of 20 mM severely decreased the growth rate of YNN348 (*rnr2-314*) while having no effect on YNN347 (*RNR2*). If the drug plates were allowed to incubate for longer periods, more clones were scored as surviving. For example, with the 20 mM dose for YNN348 and the 100 mM dose for YNN347, microcolonies were observed after 5 days but were not counted as survivors. Microcolonies were not observed for the *rnr2-314* mutant strain on the 100 mM plates even after 10 days of incubation, suggesting that this dose is strictly lethal.

The *rnr2-314* mutant displayed a higher sensitivity than did the wild type to MMS at the highest doses (Fig. 5B). We infer from the increased sensitivity of the *rnr2-314* mutant to HU that there is likely to be less ribonucleotide reductase activity under the induced conditions. The basal level of expression of *rnr2-314* supports this explanation (see below), although an increased reactivity of the mutant enzyme with HU cannot be ruled out. Thus, normal induced levels of ribonucleotide reductase activity may be needed for efficient repair of MMS-generated DNA damage. More extensive survival analysis with a variety of damaging agents will be needed to determine the precise role of *RNR2* in DNA damage repair.

Strains bearing the *rnr2-314* allele are partially constitutive for the damage response pathway and are hyperinducible by HU but not MMS. To investigate the role that *RNR2* may play in its own regulation, the dose responses of the *RNR2* regulatory region to MMS and HU were assayed in wild-type and *rnr2-314* backgrounds (Fig. 6). The basal level of β-galactosidase activity was 10-fold higher in YNN350 (*rnr2-314*), indicating that the damage response pathway may be partially constitutive in this mutant. This finding suggests that the activity of the mutant enzyme is reduced relative to that of the wild-type enzyme. The dose response to HU also was altered in the YNN350, showing a much larger increase in β-galactosidase activity at doses of 0.16 to 4 mM relative to the wild-type level. This result is consistent with the hypersensitivity to HU displayed by the mutant. However, the dose response to MMS appeared to be nearly normal, allowing for the initially high basal level in the *rnr2-314* background. These data suggest that the *RNR2* gene product is important for resistance to HU and that it can alter the sensitivity of the sensory pathway for induction by HU.

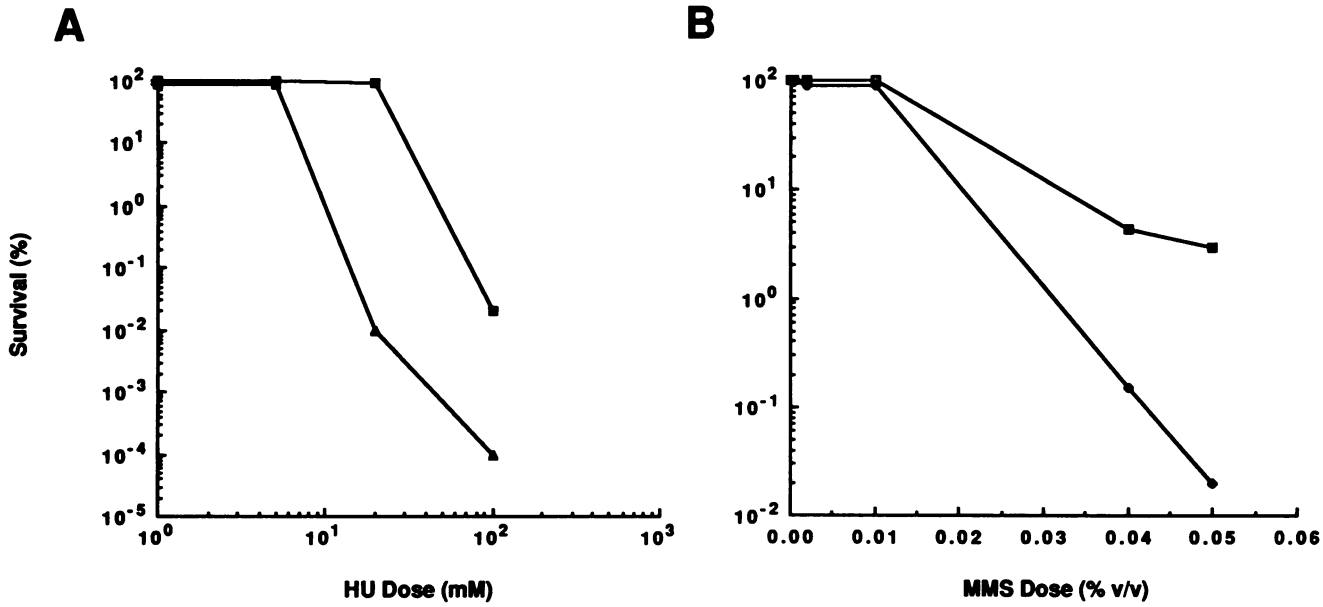

FIG. 5. Measurement of the survival of strains YNN347 (RNR$^+$) (□) and YNN348 (*rnr2-314*) (▲) on medium supplemented with HU (A) or MMS (B). Cells in early log phase were plated on YPD plates supplemented with the indicated concentrations of HU and MMS. Surviving colonies were counted and expressed as a percentage of control culture survival for each strain as described in Materials and Methods.

Although needed for efficient repair of high doses of MMS, *RNR2* does not appear to be directly involved in transmitting the damage signals produced by MMS to the cellular transduction machinery.

Overproduction of the *RNR2* protein does not affect its response to DNA damage. Since mutations in *RNR2* result in increased *RNR2* expression, it seemed possible that if *RNR2* acts as a direct repressor of its own synthesis or dampens the induction signal by providing a feedback function, then overproduction of the protein might lead to an altered regulatory response. However, overproduction of the *RNR2* protein had no effect on the induction of the *RNR2-lacZ* fusion by HU or MMS (data not shown), and therefore autoregulation by *RNR2* is unlikely to be the primary regulatory mechanism.

Mutations in the *RAD4* gene produce a hyperinducibility phenotype for *RNR2* induction by 4-NQO but not MMS. One possibility for the mechanism of induction of *RNR2* by DNA damage is as follows. As damaged DNA is acted on by excision repair processes, dNTP levels drop as a result of repair synthesis, and a nucleotide starvation signal is generated, causing the induction of *RNR2*. If this were the case, blocking the excision repair process by mutation should also block repair synthesis and the resulting depletion of nucleotides. Alternatively, if the damage inducibility of *RNR2* results from a direct sensing of the DNA damage by the sensory network of the cell, then a strain defective in excision repair should appear to have more damage than does the wild type, resulting in hypersensitivity for induction by DNA-damaging agents that produce lesions known to be

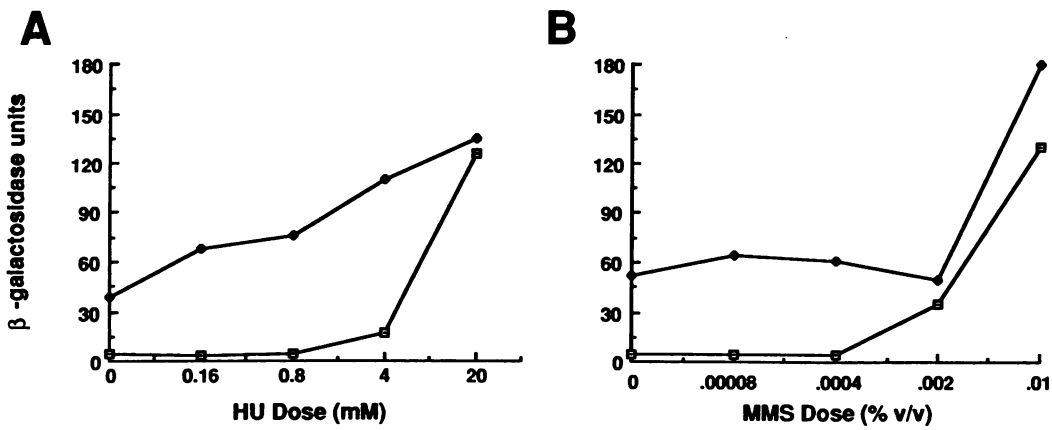

FIG. 6. Dose response of *RNR2-lacZ* levels after HU and MMS treatment in RNR2$^+$ and *rnr2-314* mutant cells. In the course of the analysis of the *RNR2* promoter (S. J. Elledge and R. W. Davis, Mol. Cell. Biol., in press), a derivative, pNN405, was created that substituted the upstream regulatory region of *RNR2* for the *CYC1* upstream activation sequence of pLG312 (14, 15). This construct allows the regulatory region of *RNR2* to control the expression of a *CYC1-lacZ* fusion gene. pNN405 was found to be inducible over a 50-fold range, whereas pNN403 gave only a 10- to 15-fold induction in response to DNA damage or perturbation of DNA replication. Because of its increased sensitivity, pNN405 was used in this experiment. Strains YNN349 (RNR$^+$) (□) and YNN350 (*rnr2-314*) (◆) were treated with the indicated concentrations of HU (A) or MMS (B) for 4 h, after which β-galactosidase activities were determined.

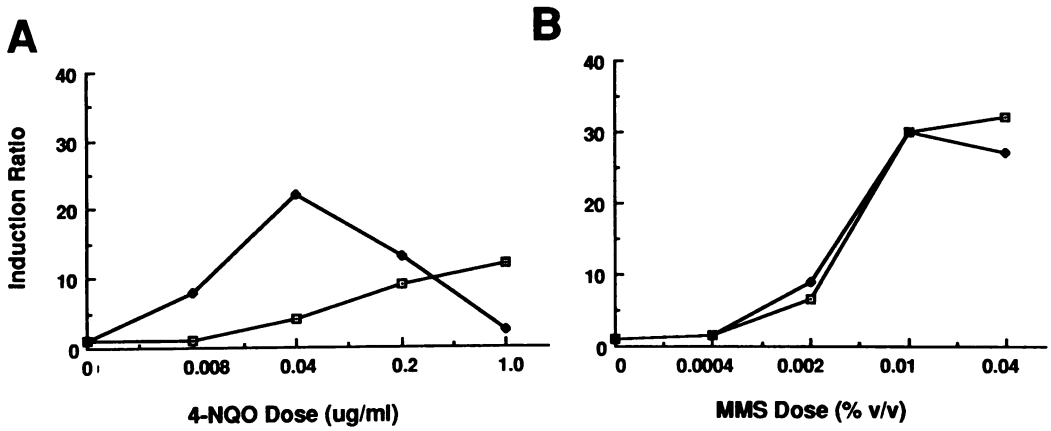

FIG. 7. Dose response of *RNR2-lacZ* levels after 4-NQO and MMS treatment in RAD$^+$ and *rad4-2* mutant cells. Strains YNN353 (RAD$^+$) (□) and YNN354 (*rad4-2*) (◆) were treated with the indicated concentrations of 4-NQO (A) or MMS (B) for 4 h, after which β-galactosidase activities were determined.

processed by the excision repair pathway. These hypotheses were tested by measuring the dose response of a wild-type strain, YNN353, versus that of an isogenic *rad4-2* mutant strain, YNN354, which is blocked in the excision repair pathway. The inducing agents used were 4-NQO, a UV-mimetic agent that causes lesions known to be repaired by the excision repair pathway (18), and MMS, a methylating agent that produces DNA damage that is repaired by a pathway other than excision repair (12). YNN354 (*rad4-2*) showed a hyperinducibility phenotype in response to 4-NQO relative to the phenotype of the RAD$^+$ control, YNN353 (Fig. 7A), but showed a normal response to MMS (Fig. 7B). These data suggest that the simple model of nucleotide depletion as the sole induction signal is incorrect and that the *RNR2* regulatory region can respond to a signal directly linked to DNA damage.

DNA damage induction of *RNR2* is cell autonomous. Yeast cells are capable of transmitting biochemical information to one another concerning their physiological state, e.g., the status of mating type, via the secretion of diffusible peptide hormones (4). It seemed possible that yeast cells may also be able to communicate the presence of a stress response from a cell that is experiencing stress to a cell that is not, as is the case for plants (26). To search for this type of interaction, we took advantage of the hypersensitivity for *RNR2* induction of *rad4-2* mutants in the presence of 4-NQO and measured the ability of RAD$^+$ cells to sense the damage stress response produced in *rad4-2* cells. The assay strain YNN353 (containing pNN405) was mixed with an equal number of cells of either SX46 (RAD$^+$) or SX46 *rad4-2*, and a dose-response analysis for the induction of β-galactosidase by 4-NQO was determined. However, the presence of cells bearing the *rad4-2* mutation had no effect on the ability of the assay strain to respond to damage (data not shown). Although this result does not rule out the ability of yeast cells to communicate the experience of stress to one another, it does suggest that in the case of *RNR2*, with respect to these experimental conditions, the DNA damage response is cell autonomous.

DISCUSSION

RNR2 is a member of a family of genes whose activities are cell cycle regulated and that are transcriptionally induced in response to the stress of DNA damage. To facilitate our analysis of the sensory mechanisms involved in this response, a gene fusion was constructed that resulted in a protein fusion between *RNR2* and *lacZ*. This fusion faithfully represented the transcriptional regulation previously observed for the *RNR2* mRNA (9, 17).

DNA damage appears to be the stimulus for induction of *RNR2*, but the precise nature of the inducing signal(s) remains elusive. *RNR2* is induced in response to a wide variety of agents that either damage DNA directly through chemical modification or induce stress by blocking DNA replication. Induction of *RNR2* is specific to the DNA damage stress response pathway(s); treatments causing thermal stress or disruption of protein synthesis do not cause induction of *RNR2*. Either of two basic models could account for specific induction of *RNR2* by DNA damage: (i) feedback regulation in response to depletion of the deoxyribonucleotide pools by repair processes or (ii) direct induction in response to DNA damage. The first model is supported by the induction by methotrexate (TTP starvation) and HU (depletion of all dNTPs). This interpretation is complicated by the fact that starvation for any nucleotide can result in a general stress signal by blocking DNA replication. Lammers and Follmann (25) characterized the yeast ribonucleotide reductase activity in a *tlr tmp* strain that is thymidylate permeable and requires thymidylate for growth. They observed induction of ribonucleotide reductase enzyme activity when cells were starved for thymidylate or in the presence of excess thymidylate, which suggests that an imbalance in the nucleotide pools can trigger the stress response. Alternatively, a secondary consequence of the imbalance could be responsible for the induction signal. For example, overproduction of one particular dNTP might cause starvation for a second dNTP by altering the normal substrate specificity of ribonucleotide reductase via allosteric interactions. A mechanism of this nature has been suggested as a possible explanation for the severe phenotype of mutations in the human adenine deaminase gene which lead to an overproduction of dATP (23).

The strongest evidence that the presence of damaged DNA is itself responsible for the induction signal is the fact that *rad4-2* mutants show hyperinducibility for induction of *RNR2* by 4-NQO. If depletion of the deoxyribonucleotide pools by excision repair synthesis causes induction of *RNR2*, then blocking excision repair should block induction of *RNR2*. It does not, which suggests that the presence of

damage is more directly responsible for producing the induction signal. This result also suggests that chemical damage to free nucleotides, which occurs when cells are exposed to agents that modify DNA, is unlikely to be solely responsible for generation of the inducing signal, since *rad4* mutants should not affect this process.

A number of genes appear to be involved in sensing and responding to the stress of DNA damage. Induction of *RNR2* is altered by the presence of a mutation in *RAD4* and by one in *RNR2* itself. At least one other pathway must exist, because neither of these mutants appears to alter the response of *RNR2* to MMS. Although perturbation of *RNR2* function produces a partially constitutive damage response, *RNR2* is not autoregulatory because overproduction of *RNR2* does not affect its response to DNA damage. The *DDR* genes also show a complex pattern of altered regulation in mutant backgrounds (30). The most likely explanation for these complex patterns of regulation is that perturbation of any of a number of normal cellular functions involved in the synthesis, repair, and maintenance of DNA can generate a stress signal(s), resulting in induction of the stress response.

In addition to understanding the molecular circuitry involved in *RNR2* regulation, it is also important to understand the physiological significance of this regulation. To accomplish this, two questions must be answered. First, what role does *RNR2* play in the cellular response to DNA damage? The sensitivity of *rnr2-314* to MMS shows that full *RNR2* function is needed for optimal repair of methylation damage. If we assume that an increase in ribonucleotide reductase activity results in the maintenance of larger dNTP pools, then increasing the dNTP pools results in maximally efficient repair. This leads us to the second question: why is ribonucleotide reductase inducible by DNA damage, or what are the advantages of increasing dNTP availability? Two models could account for the need to provide elevated ribonucleotide reductase activity. In the first model, higher levels of dNTPs provide a preventative repair function. When cells are exposed to agents that modify DNA, chemical damage to free nucleotides also occurs. Little is known about the incorporation of these damaged nucleotides into DNA. An intriguing possibility is that the role of increased ribonucleotide reductase activity in response to chemical damage is to flood the cell with undamaged dNTPs to prevent incorporation of damaged dNTPs. In the second model, DNA damage induction is needed to provide heterochronic expression of the *RNR* gene family. Since the activity of ribonucleotide reductase is cell cycle regulated, the capacity to synthesize dNTPs is reduced outside of S phase. This could result in a reduced capacity to carry out various repair processes outside of S phase. Thus, in the second model, the role of *RNR2* induction (as well as induction of other genes with cell cycle-regulated activities) is to provide dNTPs for repair outside of S phase.

Organisms do have a specific cell cycle response to DNA damage. Bacteria and animal cells respond to damage by delaying cell division. In eucaryotes, DNA damage induces arrest in the G2 phase of the cell cycle, after DNA replication and before mitosis (5, 47). G2 arrest is a physiological response aimed at preventing the molecular catastrophe of attempting segregation of incompletely replicated chromosomes. Cells blocked in this stage of the cell cycle may well enjoy the availability of many of the enzymes involved in nucleic acid metabolism that are S phase specific. However, it must also be advantageous to repair DNA damage before DNA replication to prevent the fixation of mutations as well as the production of stalled replication forks. If this is true, then the stress response to DNA damage should also function before DNA replication. This is in fact the case, because cells arrested in G1 are capable of inducing *RNR2* transcription in response to treatment with HU or MMS. *RAD54* is also inducible in G1 (7). These data suggest that the sensory pathway that detects DNA damage can operate outside of S phase, thus verifying a necessary prediction of the second model mentioned above. It should be noted that these two models are not mutually exclusive and may both contribute to the physiological significance of *RNR2* inducibility.

Our data are consistent with two possible mechanisms of *RNR2* induction in response to DNA damage: (i) alleviation of negative regulation and (ii) posttranslational modification of a positive activator. The fact that the stress response generated by 4-NQO is independent of protein synthesis suggests that the induction of *RNR2* does not rely on the de novo synthesis of a positive activator. In a separate work (S. J. Elledge and R. W. Davis, in press), we have characterized the *cis*-acting sequences in the *RNR2* promoter that mediate the DNA damage induction. This analysis has identified both positively and negatively acting elements and cellular factors that interact with these sequences. Circumstantial evidence suggests that the alleviation of repression is the likely mechanism of induction. The goal of this work has been to provide a deeper understanding of the response of *RNR2* to DNA damage in order to facilitate the isolation of the genes that mediate this response. This combination of a general analysis of the response with the characterization of *cis*-acting elements that mediate it has set the stage for the identification of mutations in genes that are directly involved in sensing and transducing the DNA damage stress response.

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