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Upstream Regulatory Sequences of the Yeast *RNR2* Gene Include a Repression Sequence and an Activation Site That Binds the RAP1 Protein

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The small subunit of ribonucleotide reductase in *Saccharomyces cerevisiae* (*RNR2*) was induced 3- to 20-fold by a variety of DNA-damaging agents. Induction of the *RNR2* transcript by at least one of these agents, methyl methanesulfonate, did not require protein synthesis. To identify sequences involved in the regulation of *RNR2*, we introduced deletions upstream of the transcription start site. Sequences required for induction were contained within a 200-base-pair region that could confer methyl methanesulfonate inducibility on the heterologous *CYC1* promoter. This region contained a repression sequence and at least two positive activation sites. One of these activation sites bound RAP1, a protein known to associate with mating-type silencers and the upstream activation sequences of a number of genes. The behavior of deletions of the repression sequence suggests that induction of *RNR2* may occur, at least in part, through relief of repression.

In *Saccharomyces cerevisiae*, the small subunit of ribonucleotide reductase, an enzyme that converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates for DNA synthesis (54), is transcriptionally induced in response to the DNA-damaging agents methyl methanesulfonate (MMS [24]) and nitroquinoline oxide (NQO [8]). Similarly, this enzyme is induced in *Escherichia coli* by chemicals that damage DNA and also is induced in mutants that inhibit DNA replication (11, 12, 18). Regulation of ribonucleotide reductase therefore may be related to processes of DNA repair or replication. We have begun to examine the mechanism of this response in yeast cells by identifying chemicals that induce *RNR2*, by showing that induction occurs in the absence of new protein synthesis, and by characterizing upstream sequences and DNA-binding proteins required for induction.

Using an *RNR2-lacZ* fusion to measure *RNR2* expression, we have found that various DNA-damaging agents, several of which cause different DNA lesions, induce the *RNR2* gene. By deletion analysis, we have identified upstream sequences involved in both activation and repression of *RNR2* transcription. One activation sequence binds RAP1, a protein that may control the activity of several constitutively expressed genes, including those encoding translational proteins (43, 60) and the glycolytic enzymes pyruvate kinase (39) and enolase (58). The RAP1 protein also binds the HMR E and HML E silencer regions in yeast cells (48), telomeres, and the promoter regions of the genes for several enzymes involved in cell growth (4). The deletion analysis presented here suggests that either the RAP1-binding site or a separate activation site suffices to support *RNR2* expression. Removal of a repression region increases uninduced expression severalfold but does not increase induced expression proportionally. These observations suggest that a repressor prevents the action of positive activators in the uninduced state and that the mechanism of *RNR2* induction may involve derepression.

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MATERIALS AND METHODS

Strains, growth, and transformations. *S. cerevisiae* TD4 (*MATa his4-519 ura3-52 leu2-3,112 trp1-289*) and F808 (*MATa GAL1*$^+$ *leu2-3,112 his4-519 ade1-100 ura3-52*) were obtained from B.-K. Tye, and strain BJ2168 (*pep4-3 prc1-407 prb1-1122 ura3-52 trp1 leu2 gal2*) was obtained from P. Sorger (50). *E. coli* Y1090 (62) was from Promega Biotec (Madison, Wis.). Yeast strains were grown at 30°C in either rich medium consisting of yeast extract, peptone, and glucose (YEPD) or in minimal medium containing yeast nitrogen base supplemented with amino acids and glucose or galactose as a carbon source. Yeast was transformed by the lithium acetate method (25), and *E. coli* was transformed as described previously (34).

Enzymes and chemicals. Enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) unless otherwise indicated. Primers for sequencing were obtained from New England BioLabs. MMS was from Eastman Kodak Co. (Rochester, N.Y.). Hydroxyurea (HU), NQO, bleomycin sulfate (BLE), cycloheximide (CH), *o*-nitrophenylgalactopyranoside (ONPG), poly(dI-dC), diethyl pyrocarbonate, phenylmethylsulfonyl fluoride, and DNase I were from Sigma Chemical Co. (St. Louis, Mo.). The UV light from a 254-nm lamp (Thomas Scientific, Swedesboro, N.J.) was measured with a Spectroline intensity meter from Fischer Scientific Co. (Rochester, N.Y.). Hybond messenger affinity paper and α-^{32}P-labeled deoxynucleoside triphosphates or [γ-^{32}P]ATP (3,000 Ci/mmol) were from Amersham Corp. (Arlington Heights, Ill.). Biotinylated goat anti-mouse immunoglobulin G, streptavidin conjugated to horseradish peroxidase, 4-chloro-1-naphthol, and protein assay dye reagent concentrate were from Bio-Rad Laboratories (Richmond, Calif.).

β-Galactosidase assays. Overnight cultures were diluted 1:100 into fresh medium and grown at 30°C to early log phase (optical density at 600 nm [OD_{600}] of 0.2 to 0.6), at which time the culture was split and grown further with or without an inducing agent. Samples were removed from the growing culture and assayed for β-galactosidase activity after cell

permeabilization (61). β-Galactosidase activity was calculated from the following equation: units = 1,000 (OD_{420} − CF × OD_{600})/time × volume × *OD_{600}, where OD_{600} and OD_{420} refer to the reaction mixture, *OD_{600} is the optical density of the cell culture at the time of assay, and CF is a correction factor for light scattering due to cells in the reaction mixture. Since the usual scattering correction factor of 1.75 was derived for *E. coli* (37), we determined it for yeast cells by performing the assay on various concentrations of cells containing no β-galactosidase. The correction factor was different for cells treated with MMS (1.44) than for untreated cells (1.19), presumably because MMS arrests yeast at the S/G2 border, thus allowing cells to enlarge without dividing (28). To verify this correction factor, we also assayed induction ratios for various *RNR2* deletions as well as for a β-tubulin–*lacZ* fusion by making crude extracts and determining specific activities (activity per mass of protein [42]). The induction ratios obtained for the permeabilized cell assay by using the derived correction factors were similar to the ratios obtained by measuring specific activities. All assays were done in duplicate. Measured β-galactosidase activities varied less than 5% for large values (>8 U), less than 10% for values of between 2 and 8 U, less than 30% for values of between 1 and 2 U, and as much as 100% for very small values (<1 U).

Nucleic acid preparation and hybridization. Plasmid DNA was prepared by the alkaline extraction method or the boiling method (34). When further purification was required, DNA was precipitated by polyethylene glycol (31). Yeast chromosomal DNA was prepared as described by Sherman et al. (46).

Total yeast RNA was isolated from cells washed in cold H_2O containing 0.2% diethyl pyrocarbonate. Pelleted cells were suspended in 1/3 to 1/10 volume of RNA extraction buffer (200 mM lithium chloride, 10 mM Tris chloride [pH 7.5], 10 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 0.2% diethyl pyrocarbonate). One-half volume of 0.45-mm-diameter glass beads (VWR Scientific, Rochester, N.Y.) was added, and the cells were lysed by high-speed vortexing in several 30-s periods interrupted by incubations on ice. The lysate was extracted with phenol-chloroform-isoamyl alcohol (1:1:0.04 [vol/vol/vol]), and nucleic acid was precipitated from the aqueous phase with 3 volumes of 95% ethanol. RNA was quantitated by staining with ethidium bromide and comparing the intensity of fluorescence with standards containing a known amount of tRNA similarly stained (34).

For Northern (RNA) blotting, RNA was electrophoresed through a 1.2% formaldehyde gel in morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) and transferred to Gene Screen Plus (Dupont, NEN Research Products, Boston, Mass.) as recommended by the supplier. The probe for hybridization was a 0.9-kilobase-pair *Hin*dIII fragment of *RNR2* labeled by nick translation with [α-^{32}P]dCTP (34).

S1 nuclease mapping. The DNA probe for S1 mapping was a ~280-base-pair fragment that was 5′ end labeled at the *Nsi*I site of *RNR2* (see Fig. 4) with T4 polynucleotide kinase and [γ-^{32}P]ATP (34). The labeled DNA was digested with a second enzyme, *Bst*XI (position −200) to generate a fragment that was labeled at only one end. The probe was electrophoresed through a strand separation gel, and the labeled single-stranded DNA was isolated as described previously (36).

Total RNA was isolated from cells with or without a multicopy plasmid carrying the *RNR2* gene, YEp13rnr (24), as earlier described. Cells carrying the plasmid were grown in minimal medium without uracil for plasmid maintenance, and cells without the plasmid were grown in rich medium. At early log phase, 0.01% MMS was added to half the culture, and growth continued for 6 h (with plasmid) or 9 h (without plasmid). Poly(A)$^+$ RNA was isolated by using Hybond messenger affinity paper as recommended by the supplier.

The 5′-end-labeled DNA probe (5,000 to 20,000 cpm per reaction) was then combined with 0.1 to 20 μg of poly(A)$^+$ RNA, and tRNA was added such that the total amount of RNA was 20 μg in each reaction. The mixture was precipitated with 0.3 M sodium acetate and 95% ethanol. Pellets were suspended in 10 μl of S1 hybridization buffer (9) and submerged at 80°C for 10 min. The samples were then removed to a water bath at 45°C and allowed to hybridize for 3 h. S1 digestion buffer (9) containing 400 U of S1 nuclease was then added, and the samples were incubated for 1 h at 14°C. The reaction was stopped with termination mix (2.5 M ammonium acetate, 50 mM EDTA, 10 μg of tRNA), and the samples were precipitated with ethanol. The pellets were suspended in formamide-dye (formamide, xylene cyanol, bromophenol blue) and loaded on an 8% polyacrylamide–7 M urea gel. The 5′ end (see Fig. 4) was localized by comparing the migration of the protected fragment with the migration of the cleavage products generated by the Maxam and Gilbert A-G reaction (see Fig. 3; 36), taking into account the faster migration (by 1.5 bases) of fragments produced by chemical sequencing than of S1 nuclease-derived fragments (35, 55).

Construction of plasmids and deletions. The *RNR2-lacZ* fusion was constructed from the ~600-base-pair *Hin*dIII-*Nsi*I fragment of *RNR2* (containing the transcriptional start site, upstream regulatory sequences, and 13 amino acids of N-terminal coding sequence; see Fig. 4) and the *lacZ* gene of plasmid pMC1871 (5). A fragment containing the *RNR2-lacZ* fusion was cloned into the vector pSZX, a derivative of pSZ58 (40) that carries the *LEU2* gene for selection in yeast cells. The resulting plasmid, p0-1Kpn, was cut at a unique *Bst*EII site in the coding sequence of the *LEU2* gene and integrated into strain TD4 in single copy (checked by Southern analysis; data not shown) at the endogenous *LEU2* locus on chromosome III (51). TD4 cells containing this integrated *RNR2-lacZ* fusion were used in the experiment of Fig. 1 except for induction with UV light. A second *RNR2-lacZ* fusion, containing about 90 additional N-terminal amino acids of *RNR2* and integrated at a different site (*Xho*I) in *LEU2*, gave results similar to those in Fig. 1; thus, the regulatory response was not significantly affected by the fusion junction, the presence of additional *RNR2* sequences, or the exact site of integration. The second fusion was used to obtain the data for UV induction in Fig. 1.

Deletions in the regulatory region of *RNR2* were constructed by removing sequences 3′ to the *Hin*dIII site (−528; see Fig. 4) by using exonuclease III and S1 nuclease. *Hin*dIII linkers were attached to the blunt ends, the DNA was digested with *Hin*dIII and *Xba*I (to cut downstream of the *lacZ* gene), and the *Hin*dIII-*Xba*I fragments were cloned into pSZX (see above) that had been cut with *Hin*dIII and *Xba*I. Endpoints of the deletions were identified by double-strand sequencing (63), and a series of these 5′ deletions was cut with *Kpn*I, a unique site in the *LEU2* gene of pSZX, and integrated in single copy at the *LEU2* locus.

Deletions from the 3′ end of the *RNR2* promoter were made by cutting plasmid p0-1Kpn near the *RNR2-lacZ* junction with *Bam*HI, digesting with exonuclease III and S1 nuclease, attaching *Hin*dIII linkers, and cutting with *Hin*dIII and *Pst*I (which cleaves upstream of the 5′ end of *RNR2*). The *Hin*dIII-*Pst*I fragments were cloned into pSZX, and the

deletion endpoints were sequenced as described above. Various 3′ deletions were then recombined with 5′ deletions by cloning the *RNR2-lacZ*-containing *Hin*dIII-*Xba*I fragments from the 5′ deletions into the *Hin*dIII-*Xba*I vector backbone carrying the 3′ deletions. This series of internal deletions was introduced into strain TD4 in single copy at the *Bst*EII site of the *LEU2* gene.

The *RNR2-CYC1-lacZ* fusions were constructed from a derivative of the plasmid pLG669Z, which contains the upstream sequences of *CYC1* fused to the *lacZ* gene as well as the *URA3* gene and 2μm replication origin sequences for selection and growth (15). The derivative, pLG669ZKpn (a gift of X. Hua), was made by digesting pLG669Z with *Sma*I and *Xho*I to remove the regulatory upstream activation sequences UAS1 and UAS2 (14); the *Xho*I site was then filled in, and the plasmid was recircularized with a *Kpn*I linker. Two 3′ deletion plasmids with endpoints at −312 (pRCZ20β) and −157 (pRCZ10α) were cloned into the *Xho*I and *Kpn*I sites of pLG669ZKpn, thus placing *RNR2* sequences in the same region as the *CYC1* UASs. These plasmids, together with the pLG669ZKpn control without an insert, were transformed into strain TD4 and assayed for β-galactosidase activity.

The λgt11 lysogens carrying the *RAP1* gene were reconstructed from plasmid pRAP, which contains an *Eco*RI-*Eco*RI fragment of the *RAP1* gene in pUC13 (a gift from D. Shore, derived from the original λgt11 isolate 11.4 [47]) and λgt11 dephosphorylated arms (Promega Biotec). Those λgt11 DNAs containing a *RAP1* insert in both orientations were lysogenized in *E. coli* Y1090 along with the λgt11 vector as a control.

Plasmid pGALRAP was constructed from plasmid pRAP described above. Exonuclease III and S1 nuclease were used to delete *RAP1* sequences upstream of the ATG translational start site (the deletion endpoint is at position 590 in Shore and Nasmyth's sequence of *RAP1* [47]). The deleted *RAP1* gene was cloned into plasmid YIPGAL, a derivative of the integrative plasmid YIP352 (22) that contains the intergenic regulatory region of *GAL1* and *GAL10* from pCGS109 (from J. Schaum [Collaborative Research, Inc., Bedford, Mass.] via B.-K. Tye). The UAS for galactose induction is included in the sequence cloned, as is the +1 transcription start of the *GAL1* gene. The resulting plasmid, pGALRAP, was digested at a unique *Nru*I site in the coding sequence of *RAP1* and integrated at the *RAP1* locus in the multiply protease-deficient strain BJ2168.

Yeast whole-cell extracts and λgt11 lysates. Yeast whole-cell extracts of BJ2168 were prepared essentially by the method of Bram and Kornberg (2), with some modifications noted below. A saturated culture of BJ2168 was subcultured 1:50 and grown to an OD_{600} of ~0.5 in rich medium. MMS (0.01%) was added to half of the culture, and the cells were grown for an additional 8 to 9 h at 30°C. The cells were then spun down, washed with cold H_2O, and stored at −70°C. BJ2168 cells containing the integrated copy of the *GAL1-RAP1* fusion (plasmid pGALRAP) were grown in minimal medium without uracil in the presence of glucose to saturation. BJ2168(pGALRAP) cells were then subcultured 1:50 into minimal medium plus 2% glucose or 1:25 into minimal medium plus 2% galactose and allowed to grow at 30°C to an OD_{600} of 2 to 5. Cells were then spun down, washed in cold H_2O, and stored at −70°C.

To determine whether strain BJ2168 contains the wild-type regulatory genes required for galactose induction, plasmid pCGS286 (from J. E. Mao, Collaborative Research), which carries the *GAL1* promoter fused to the *lacZ* gene, was transformed into strains BJ2168 and F808, a strain known to be GAL^+, and β-galactosidase expression was compared in the presence of glucose and galactose in these two strains. Both BJ2168 and F808 showed no β-galactosidase activity when grown in glucose and comparable activity after 11 h of growth in galactose, indicating that BJ2168 has the relevant galactose regulatory genes. (BJ2168 carries a *gal2* mutation, a mutation in galactose permease that slows galactose uptake; however, this mutation does not affect *GAL1* expression in these experiments because BJ2168 (pGALRAP) cells were grown to late log phase before harvesting for protein extraction, which allowed time for galactose utilization.)

BJ2168 and BJ2168(pGALRAP) cells were disrupted in buffer A (without leupeptin and pepstatin) as described previously (2) except that a vortexer was used instead of a bead beater, since small volumes of cells were disrupted. Ammonium sulfate was added to 0.4 M, and after a 30-min extraction at 4°C, the supernatant was spun at 340,000 × *g* for 2 to 3 h. The supernatant was dialyzed twice against 10 to 15 volumes of buffer A, and the protein concentration was determined with the Bio-Rad protein assay dye reagent as recommended by the supplier.

λgt11 lysates were prepared by the method of Shore and Nasmyth (47) except that the only protease inhibitor used was phenylmethylsulfonyl fluoride. Protein concentrations were determined as described above, and extracts were stored at −70°C.

Gel electrophoresis retardation experiments. The DNA fragment used to visualize protein-DNA complexes consisted of *RNR2* sequences from −306 to −527, labeled by filling in restriction-cut ends with [α-^{32}P]dATP. The fragments used as competitor were a ~260-base-pair fragment containing *RNR2* sequences from −306 to −527, which includes the RAP1-binding element (RBE) and a ~160-base-pair fragment containing *RNR2* sequences from −409 to −527, which lacks the RBE (see Fig. 4).

Approximately 1 ng of probe was mixed in a total volume of 20 μl with 9 or 22.5 μg of yeast whole-cell extract untreated or treated with MMS or with 10 μg of a λgt11 lysate in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 60 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM $MgCl_2$, 11% glycerol, and 1.5 to 2 μg of poly(dI-dC). Cold competitor was added in some cases to 100-fold molar excess over the probe. The mixture was incubated 10 to 15 min at room temperature and electrophoresed through a 2.4% agarose gel in 22.5 mM Tris borate (pH 8.3) and 0.63 mM EDTA. The gel was dried and exposed to Kodak X-Omat AR film (Eastman Kodak Co.) at −70°C with a Cronex Lightning-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The specificity of the protein-DNA interaction observed was analyzed by comparing the ability of nonspecific DNA [poly(dI-dC)] and specific DNA (RBE-containing fragment) to compete for binding to the RBE fragment. Cold RBE-containing fragments competed for binding about 100-fold better than did poly(dI-dC) sequences (data not shown).

Immunoblotting. A 25-mg sample of yeast extract prepared as described above was electrophoresed through an 8% polyacrylamide–SDS gel (acrylamide/bisacrylamide ratio of 30:1) and transferred electrophoretically to nitrocellulose (56). The nitrocellulose was blocked for 30 min with 3% gelatin in Tris-buffered saline (50 mM Tris chloride [pH 7.5], 150 mM sodium chloride) and incubated for 3 h in a 1:2,500 dilution of mouse polyclonal antibody to the RAP1 protein (a

gift from D. Shore [47]). The blot was washed twice for 10 min in Tris-buffered saline, incubated for 1 h in biotinylated goat anti-mouse antibody (diluted 1:1,000), washed again, and finally incubated for 1 h in streptavidin-horseradish peroxidase (diluted 1:1,000). After two more Tris-buffered saline washes, the blot was developed by using 4-chloro-1-naphthol as recommended by the supplier. The relative amounts of protein in the bands of Fig. 8 were determined by densitometric scanning of a stained immunoblot in which samples were diluted to obtain a linear standard curve (data not shown).

Southwestern (DNA-protein) blotting. Protein extracts were immunoblotted to nitrocellulose in 25 mM Tris (pH 8.3)–190 mM glycine (no methanol) (20). The nitrocellulose filters were then blocked for 60 min at room temperature with BLOTTO and washed twice with TNE-50 (49). Protein bound to the filters was denatured and renatured (6), and the filters were incubated for 1 h at room temperature in TNE-50 containing 10 μg of poly(dI-dC) per ml and either 2×10^4 to 4×10^4 cpm of end-labeled probe per ml (for the λgt11 lysates) or 5×10^5 to 6×10^5 cpm of end-labeled probe per ml (for the yeast extracts). The filter was then washed as described previously (49) and exposed to film at −70°C.

DNase I footprinting. A 294-base-pair *Hin*dIII-*Bst*XI fragment from the −479 5′-deletion plasmid that had been end labeled with Klenow fragment and [α-^{32}P]dATP was used for DNase I protection mapping. Approximately 150,000 cpm of fragment labeled at the *Hin*dIII end, 1 μg of poly(dI-dC), buffer A (without pepstatin and leupeptin), and protein were mixed in 20 μl (3). Samples were incubated at room temperature for 10 min, and 5 μl of DNase I solution containing 3.9 mU (Kunitz milliunits) for no added protein, 9.6 mU for 0.5 and 2 μg of yeast protein, or 19.3 mU for 11 μg of yeast protein was added; the mixture was incubated at room temperature for 1 min. More DNase I was used for more added protein, since the extracts appeared to contain an inhibitor of DNase I. The reaction was stopped by the addition of 25 μl of stop buffer containing 1% SDS, 20 mM EDTA, 200 mM NaCl, and 20 μg of glycogen per ml as carrier, followed by phenol extraction and ethanol precipitation. The pellets were resuspended in formamide-dye and electrophoresed through 6% acrylamide–7 M urea gels.

RESULTS

Induction of *RNR2* by DNA-damaging agents. The ability of DNA-damaging agents other than MMS to induce *RNR2* was tested by assaying β-galactosidase activity in cells carrying a *RNR2-lacZ* fusion that were grown in the presence of different agents. The *RNR2-lacZ* fusion was induced 18-fold by 0.01% MMS, 13-fold by 100 μg of BLE per ml, 12-fold by 50 ng of NQO per ml, 9-fold by 30 mM HU, and 3-fold by 20 J of UV light per m^2 (Fig. 1). These induction ratios were determined by dividing the induced activity (at the time of maximum induction) by the basal activity. Other DNA-damaging agents that did not significantly induce *RNR2* (less than threefold over the basal level) include mitomycin C at 50 μg/ml, methotrexate at 10 μg/ml, nalidixic acid at 50 μg/ml, N-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) at 1.25 ng/ml, coumermycin at 10 μg/ml, and ethyl methanesulfonate at 0.02%. The magnitudes of *RNR2* induction caused by these agents should not be compared quantitatively because only one dose was tested, as used in the following references: MMS, NQO, UV, and MNNG (44); BLE, mitomycin C, methotrexate, and nalidixic acid (41); and coumermycin (13). Furthermore, some chemicals may be imper-

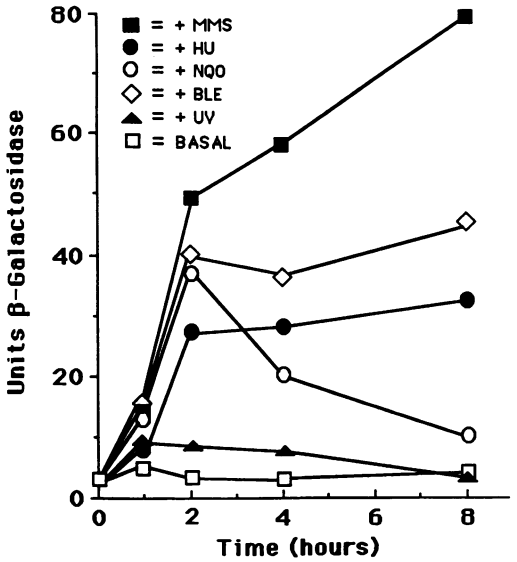

FIG. 1. Induction of the *RNR2-lacZ* fusion protein in the presence of 0.01% MMS, 30 mM HU, 50 ng of NQO per ml, 100 μg of BLE per ml, and 20 J of UV light per m^2. Cells carrying the *RNR2* promoter fused to *lacZ* and integrated at the *LEU2* locus were grown in YEPD (except cells treated with UV light, which were grown in minimal medium) in the presence of these DNA-damaging agents and assayed for β-galactosidase activity at the indicated times. Units of β-galactosidase are relative to cellular optical density as described in Materials and Methods.

meant or toxic to yeast cells. Since MMS induced *RNR2* significantly better than did the other agents, it was used in subsequent experiments to analyze the requirements of induction.

A minor portion of the increase in β-galactosidase activity of the *RNR2-lacZ* fusion may represent a general response to MMS. When the same assay was used to measure β-galactosidase activity in an actin-*lacZ* or β-tubulin–*lacZ* fusion containing the regulatory region of actin or β-tubulin, respectively, we found an increase of about 1.5-fold after MMS treatment. Similarly, the activity of a *CYC1-lacZ* fusion containing the TATA element and transcriptional start sites of *CYC1* but no *CYC1* regulatory sequences (see Fig. 6) also increased 1.5-fold with MMS. Thus, MMS appeared to cause a slight generalized increase in β-galactosidase activity (which might occur at any level at which gene expression is regulated), but the specific response of *RNR2* was clearly detectable above this background.

Induction of the *RNR2* transcript is independent of protein synthesis. The *RNR2* transcript was induced by MMS in the presence or absence of CH (Fig. 2), indicating that synthesis of new proteins is not required for induction. Densitometric scanning of the autoradiograph normalized to 26S and 18S RNAs stained with ethidum bromide showed a comparable induction of *RNR2* mRNA by MMS in the presence (5.4 OD units) and absence (5.1 OD units) of CH. The level of uninduced RNA with and without CH was about 0.5 OD units, indicating that CH did not affect uninduced levels of the *RNR2* transcript (data not shown). CH effectively inhibited protein synthesis, since there was no increase in β-galactosidase activity from the *RNR2-lacZ* fusion despite the mRNA induction.

S1 nuclease mapping of the transcription start site. The labeled DNA fragment protected from S1 nuclease digestion

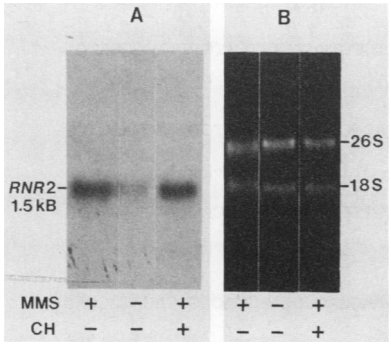

FIG. 2. Effect of CH on induction of the *RNR2* transcript. (A) Northern blot of RNA isolated from cells grown in the presence of 0.01% MMS with or without 100 μg of CH per ml. The uninduced level of RNA shown is from cells grown in the absence of MMS and CH, but cells grown without MMS in the presence of CH had the same amount of the *RNR2* transcript (data not shown). RNA was isolated from strain TD4, which was grown at 30°C to early log phase in YEPD, and split into four samples (minus MMS and CH; minus MMS, plus CH; plus MMS, minus CH; and plus MMS and CH) and then grown for an additional 8 h. (B) Same RNA gel stained with ethidium bromide before transfer. The RNAs shown are the 26S and 18S rRNAs.

by the *RNR2* transcript is shown in Fig. 3. The major 5′ end of the *RNR2* transcript mapped to an A residue (+1 in Fig. 4). This 5′ end is likely the start site of the *RNR2* RNA rather than a 3′ intron boundary, since the highly conserved consensus sequences characteristic of an intron, including the TACTAAC box, are not present (16). This transcriptional initiation site does not fit into either the TCG/AA or RRYRR class of start sites defined by Hahn et al. (17). The

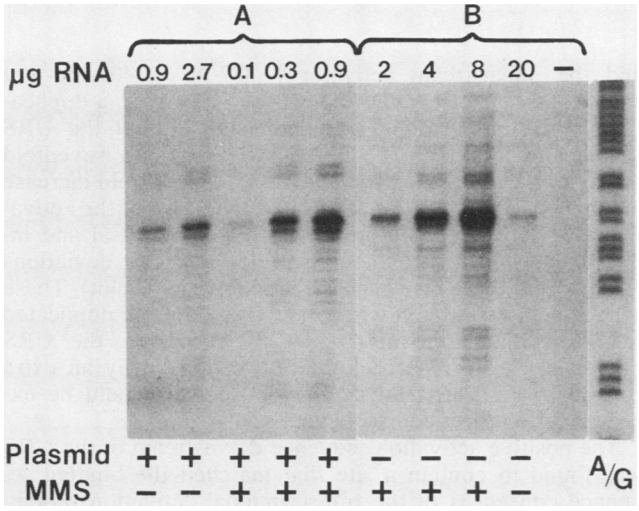

FIG. 3. S1 nuclease mapping of the 5′ ends of the *RNR2* transcript. (A) RNA isolated from cells carrying a multicopy plasmid with the *RNR2* gene grown in the absence or presence of 0.01% MMS; (B) RNA isolated from the same strain not carrying the plasmid, with or without MMS. Panels A and B (with and without plasmid) cannot be quantitatively compared directly because the gels were exposed differently. The amount of poly(A)$^+$ RNA is indicated, and the absence of a protected fragment is shown in the lane without added RNA (−). The 5′ end of the RNA was localized by comparing the protected fragment with the same labeled fragment cleaved by the Maxam and Gilbert A-G reaction.

existence of a 50-nucleotide 5′ noncoding segment is typical of yeast cells. However, the only likely TATA element is at −139 relative to +1, which is further upstream than the believed maximum distance of 120 base pairs (17, 38, 53). The measured increase in 5′ ends of the *RNR2* transcript, 15- to 39-fold after 9 h of growth with MMS (Fig. 3 and data not shown), is consistent with the previously measured 17-fold increase in steady-state mRNA levels in the presence of MMS (24). Densitometric scanning showed that the autoradiographic signal in Fig. 3 increased linearly with increasing input RNA (data not shown), indicating that the probe was in excess of the RNA and therefore that the measurement of 5′ ends was quantitative. Cells with a 2μm multicopy plasmid carrying the *RNR2* gene overproduced the RNR2 protein about 20-fold, and as expected, the presence of this plasmid caused overproduction of the 5′ ends of the *RNR2* transcript. (The number of 5′ ends in cells grown with and without the plasmid cannot be compared directly in Fig. 3 since panels A and B were exposed differently.)

Fusion of *RNR2* regulatory sequences to the heterologous *CYC1* gene. Deletions were introduced into the upstream sequences of *RNR2* to identify regions that control basal and inducible expression. When the region from −528 to −346 was deleted, most of the inducible activity was eliminated and the basal activity was reduced as well (Fig. 5), suggesting that sequences between −528 and −346 are involved in the regulation of *RNR2* expression. In agreement with this identification of the regulatory sequences, a fragment containing *RNR2* sequences from −312 to −528, placed upstream of a *CYC1-lacZ* fusion, conferred MMS inducibility on the foreign *CYC1* promoter (pRCZ20β; Fig. 6). The amount of induction of β-galactosidase activity controlled by this 217-base-pair fragment was about the same as that conferred on the *CYC1* gene by a larger fragment containing nearly all of the upstream sequences of *RNR2* from −157 to −528 (pRCZ10α; Fig. 6). This result suggests that sequences from −157 to −312 do not significantly contribute to MMS induction. However, it should be noted that the *RNR2-CYC1-lacZ* fusions are not strictly comparable to the *RNR2-lacZ* fusions, since the former are on 2μm multicopy plasmids, whereas the latter are integrated in single copy into the chromosome.

Although the basal level of expression of the *RNR2-lacZ* fusions ranged from 4 to 6 U, neither the 217-base-pair fragment from −312 to −528 (pRCZ20β; Fig. 6) nor the larger fragment from −157 to −528 (pRCZ10α; Fig. 6) conferred that level of expression on the *CYC1-lacZ* gene in the absence of MMS (0 to 2 U; Fig. 6). Other *RNR2* sequences not present in these constructs may be required for *RNR2* basal expression or the elements responsible for constitutive expression of *RNR2* may act only on the TATA elements of their own gene, as is the case for *HIS3* and *PET56* (52).

Deletions in the *RNR2* promoter identify a repression sequence and redundant activation sites. The behavior of the series of deletions extending from the 5′ end of the *RNR2* promoter toward the TATA element indicated the presence of a repression sequence between −397 and −371. This upstream repression sequence (URS) was further localized to the region between −387 and −359 by internal deletions (compare deletions −387 to −409, −371 to −409, and −167 to −359; Fig. 5). Deletion of the URS typically caused an increase in basal expression of three- to fivefold. However, the induction ratios of deletions that removed the URS were much less than the wild-type induction ratio (compare the induction ratios of deletions −355 and −528; Fig. 5), which

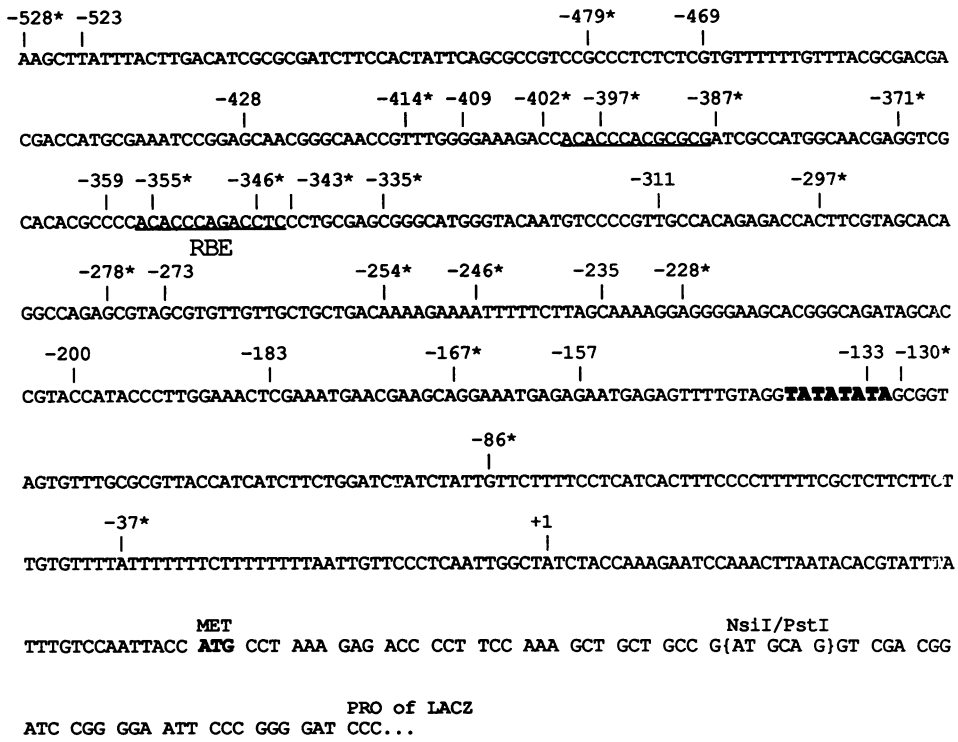

FIG. 4. *RNR2* sequence showing deletion endpoints relative to the transcription start site (+1). 5′ deletions have an asterisk; the others are 3′ deletions. The endpoints include the nucleotide indicated, which is attached to a *Hin*dIII linker 5′-CCAAGCTTGG-3′. Sequences that fit the consensus for RAP1 binding (3) are underlined, and the site that RAP1 binds to in vitro is indicated as RBE. The probable TATA element is highlighted and begins at −139. The *RNR2-lacZ* fusion begins at the *Nsi*I site of *RNR2* and is followed by a polylinker. The lacZ protein-coding region begins at the proline indicated, which is the eighth amino acid of lacZ. This sequence also shows corrections in the previously published sequence (8, 24) that were discovered when the deletions were sequenced. The changes are as follows (numbering as given in reference 24 is in parentheses): an extra C is present at −382 (146), the G and C at −284 (244) and −283 (245) are switched, the C at −280 (249) is removed, an extra G is present at −276 (252), the G and T at −271 (257) and −270 (258) are switched, the G at −43 (486) is changed from a C, the T at −33 (495) is changed from a C, the A at −8 (520) is changed from a G, the T at −6 (522) is changed from an A, and the C at +15 (542) is changed from an A.

suggests that the mechanism of *RNR2* induction might involve a release of repression at the URS.

The properties of the deletions suggested that one of two redundant activation sites was required for *RNR2* expression. One activation site was located between −428 and −469 (compare the deletion from −167 to −428 with the deletion from −167 to −469; Fig. 5), and part or all of the other site was between −355 and −346 (5′ deletions −355 and −346). Loss of both sites prevented induced expression as well as derepression through deletion of the URS (deletions −346 and −167 to −469). The two activation sites may not be completely redundant, since deletion of one or the other resulted in less basal activity than when both were present in the absence of the URS (deletion −371 to −409, which removed the URS but retained both activation sites). The spacing between the downstream activation site and the closely situated URS did not seem to be critical, since a 7-base-pair insertion (deletion −355 to −359 removed 3 base pairs, but the *Hin*dIII linker added 10 base pairs) did not significantly affect expression.

The behavior of several tandem duplications was consistent with the assignment of activation and repression functions. The basal activity of the smallest internal deletion that did not appear to remove regulatory sequences (deletion −163 to −183) was 1 to 2 U, and the induced activity was 35 U. If we take these activities as unmodified levels, duplication of the URS (−387 to −359) or duplication of the URS and the downstream activator (−387 to −340) did not significantly change *RNR2* expression. However, a duplication of the downstream activation site without the URS (duplication −371 to −340) caused a six- to sevenfold increase in basal expression and a two- to threefold increase in induced expression; in addition, duplication of the activation site between −479 and −428 increased basal and induced expression just under twofold (standard deviations indicate that these increases are probably significant). These increases in expression would be expected if the duplicated regions contain activation sites. Furthermore, the URS prevented the increased expression conferred by an extra copy of the downstream activation site, as would be expected for a repression sequence.

The positive activation sequence downstream of the URS was found to contain a site that matched the binding sequence consensus of the transcriptional activation protein RAP1 in 12 of 13 base pairs (3). Another sequence that matched the consensus in 11 of 13 base pairs is underlined in Fig. 4 and represented by an arrow in Fig. 5. However, only the site that matched in 12 of 13 base pairs bounds the RAP1 protein in vitro (see below), and it therefore is named the RBE. The RBE is likely to be one of the redundant activation sites required for increased basal expression in the absence of the URS, since a deletion from −528 to −355, which replaced the first base pair of the consensus with a linker-derived G residue that also fit the consensus, did not

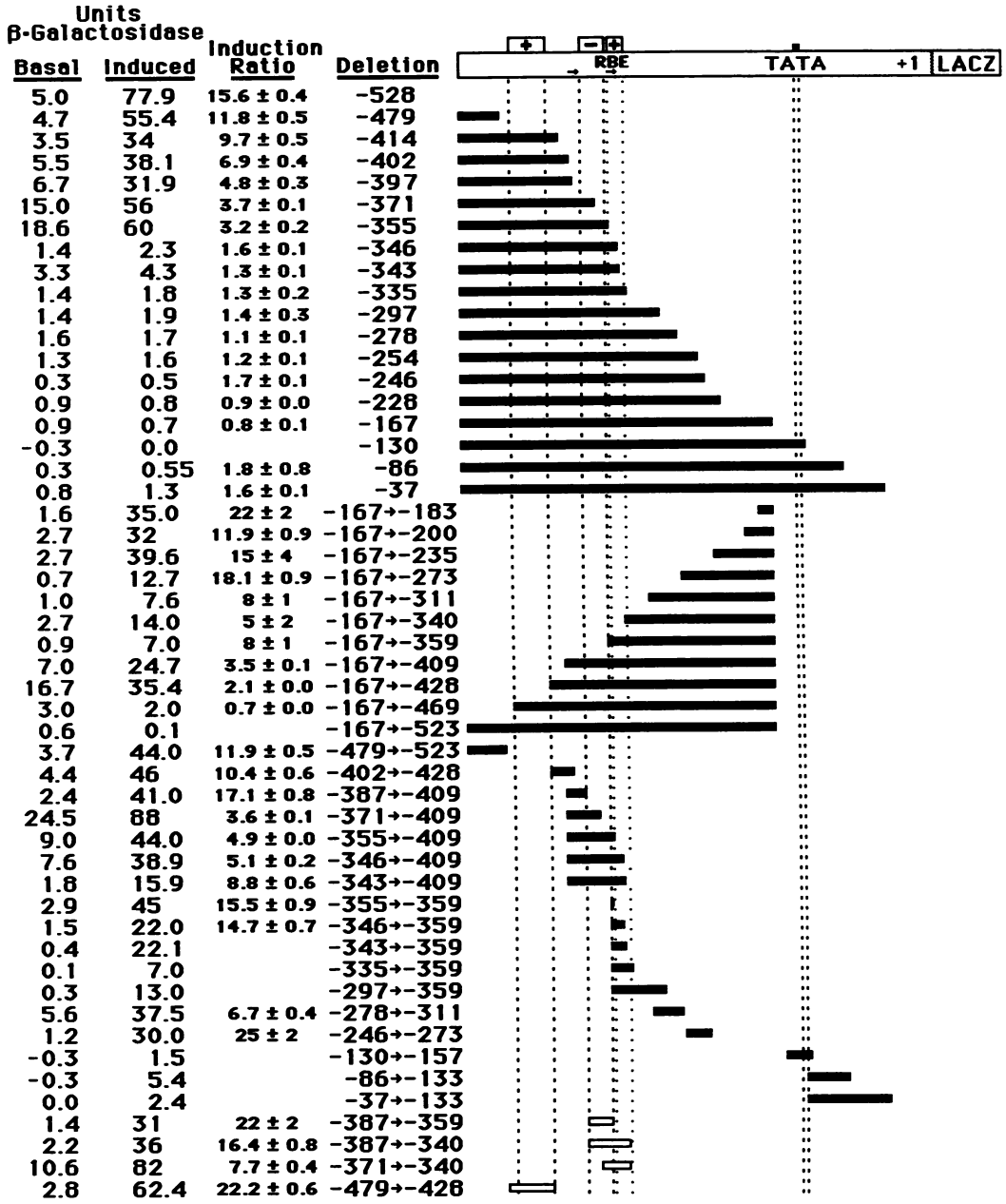

FIG. 5. Summary of 5′, 3′, and internal deletions of the *RNR2* promoter fused to *lacZ* and the basal and induced β-galactosidase activities for each construct. Induced expression was determined by assaying cells carrying the fusion construct integrated in single copy at the *LEU2* locus and grown with MMS in YEPD for 4 h. Basal expression was determined in cells grown without MMS. Some activities are less than zero because of a correction factor for the absorbance of cells in the reaction mixture (see Materials and Methods). The induction ratios include a standard deviation that was derived from the summation of the variation in each induced and basal value. Ratios determined from basal or induced activities that were very low and had a high degree of uncertainty are not shown. The deletion endpoints are inclusive of the base pair indicated. Symbols: ■, sequences deleted; □, regions duplicated in tandem relative to the diagram of the *RNR2* promoter-*lacZ* fusion shown at the top. Arrows indicate potential RAP1-binding sites; the arrow closest to the TATA element is the RBE, the sequence that RAP1 binds to in vitro. Boxes above the map indicate regions required for activation (+) or repression (−). The transcription start site is at +1, and the probable TATA element is upstream at −139.

interfere with high basal expression, whereas a deletion that removed 9 more base pairs of the consensus (deletion −528 to −346) eliminated the high basal activity. The other site that more loosely matched the consensus for RAP1 binding did not appear to affect the expression of *RNR2* by deletion analysis.

In addition to the two positive elements and the URS that clearly affected *RNR2* expression, several other regions partially decreased the ratio of induced to basal expression when deleted: sequences between −479 and −528, between −273 and −311, and between −183 and −200 (compare the wild-type induction ratio [−528 deletion] with the ratios for deletions −479, −167 to −183, −167 to −200, −167 to −273, and −167 to −311; Fig. 5). These regions may contain sites

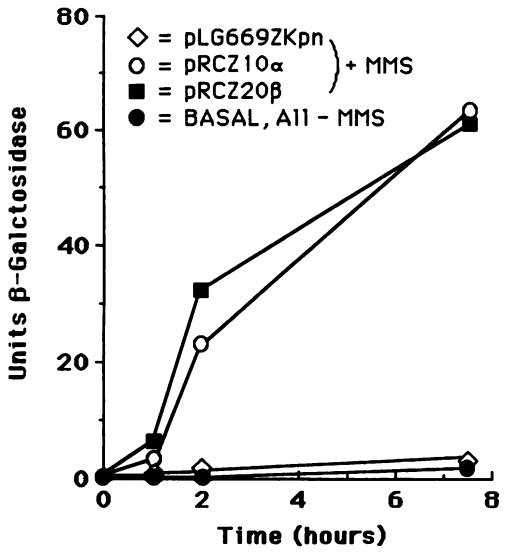

FIG. 6. Effect of *RNR2* promoter sequences on the induced expression of a heterologous gene. Plasmid pLG669ZKpn is a 2μm vector carrying the *URA3* gene and part of the *CYC1* gene fused to *lacZ*. Upstream activation sequences have been removed such that *CYC1-lacZ* expression is low. In pRCZ10α and pRCZ20β, parts of the *RNR2* promoter (−157 to −528 plus linkers and −311 to −528 plus linkers, respectively) have been inserted in place of the *CYC1* UASs. These plasmids and the control without *RNR2* or *CYC1* sequences were transformed into strain TD4. Transformants were grown in the presence of MMS in minimal medium (minus uracil for plasmid maintenance) and assayed for β-galactosidase expression. The curve for basal activity represents the activity of all three plasmid-carrying strains (pRCZ10α, pRCZ20β, and pLG669ZKpn) in the absence of MMS. The other curves represent the β-galactosidase activity detected when the indicated plasmid-carrying cells were grown in the presence of MMS.

that contribute to full induced expression. Several other internal deletions also decreased basal activity, but the effects were sporadic and could, for example, reflect a response to changes in spacing between regulatory elements.

The behavior of only one deletion failed to support the model that there is a repression sequence flanked by two activation sequences: deletion −343 to −409 removed the URS and part of the downstream activation site but had a fairly high induction ratio (~9) relative to the ratios of other deletions of the URS, which were between 3 and 5. We offer two related possible explanations of this observation. First, the URS might not be a protein-binding site but instead might be a region in which structural alterations destroy repression, for example by altering a complex DNA-protein structure that represses; a particular deletion then might leave this structure sufficiently intact to repress. Second, if the URS is a protein-binding site, other unidentified binding sites also might contribute to repressor binding, perhaps through looping; in that case, an exceptional deletion might provide a structure that allows these secondary sites to bind the repressor well enough to repress.

The probable TATA element in the *RNR2* promoter is located between −139 and −132. When the region from −130 to −157 was deleted, expression was reduced nearly to zero. A similar reduction in basal and induced expression was seen upon removal of part of the T-rich tract (−37 to −133). A T-rich tract that affects expression is also present in the upstream region of ribosomal genes that have an RBE (RPG box [43]). However, the T-rich tract may not be particularly important for *RNR2* expression, since a deletion that removed *RNR2* sequences downstream of the TATA element but upstream of the T-rich tract (−86 to −133; the T-rich tract extends from approximately −10 to −80) also significantly decreased both basal and induced expression. Therefore, specific sequences between the TATA element and the transcriptional start site or the distance between TATA and +1 may be important for *RNR2* expression.

Detection of protein-DNA complexes with *RNR2* DNA by mobility shift. A fragment of DNA containing *RNR2* regulatory sequences (from −306 to −527), as determined by deletions and *RNR2-CYC1-lacZ* fusions, was labeled, mixed with yeast extracts, and subjected to gel electrophoresis in an attempt to identify sequence-specific DNA-binding proteins. The mobility of this fragment (RBE DNA) was retarded in an agarose gel in the presence of extract (Fig. 7), suggesting that a protein or proteins bind the regulatory region. The same shift occurred when extracts made from cells grown with or without MMS were used, indicating that MMS caused no gross change in the in vitro binding activity. Complex formation was specific to sequences in this fragment because a 10-fold molar excess of the same fragment (unlabeled) competed for binding as well as did a 2,000-fold excess of poly(dI-dC) (data not shown). The specificity of the protein-DNA interaction is corroborated in Fig. 7B, in which a different segment of the *RNR2* promoter (from −335 to −182; control DNA) was labeled and included with the radioactive RBE-containing fragment. It is clear that the RBE-containing fragment was shifted preferentially over the control fragment, although the total radioactivity in each lane varied too much to allow us to conclude that none of the control DNA was shifted (Fig. 7B).

Since the fragment of DNA used in these experiments contained an RBE, it seemed likely that the RAP1 protein caused the shift in mobility. To establish this, we first showed that the protein responsible for the mobility shift bound the RBE by showing that an excess of the same fragment (unlabeled) competed for binding, but an excess of a similar fragment missing the RBE did not (Fig. 7A).

E. coli lysates from λgt11(*RAP*) lysogens synthesized the RAP1 protein (Fig. 8). When these lysates were incubated with the labeled *RNR2* fragment, a protein-DNA complex was formed that migrated to the same position as did the complexes formed in yeast extracts (Fig. 7B). This result suggested that the protein bound to the RBE in yeast cells was the RAP1 protein. The lysate of a λgt11 lysogen without the *RAP1* gene, which produced no RAP1 protein (Fig. 8), did not form a protein-DNA complex (Fig. 7B).

Overproduction of the RAP1 protein. To further implicate the RAP1 protein in DNA binding (see below), we fused the *RAP1* gene to the *GAL1* promoter so that the RAP1 protein could be overproduced. When the pGALRAP construct was integrated at the *RAP1* locus of strain BJ2168 and grown in the presence of galactose, the *RAP1* protein was overproduced 10-fold (Fig. 8). The amount of RAP1 protein in strains without pGALRAP was comparable to (but slightly less than) the amount in pGALRAP strains grown in glucose, in which the *GAL1* promoter was presumably turned off.

Identification of RAP1 binding to *RNR2* DNA by Southwestern analysis. The RAP1 protein could be denatured and renatured such that it regained DNA-binding activity, as shown by a Southwestern analysis of yeast whole-cell extracts in which RAP1 protein immobilized to a nitrocellulose filter bound to a labeled fragment of the *RNR2* promoter containing the RBE (Fig. 9). As was observed in the gel electrophoresis retardation experiments, RAP1 protein from

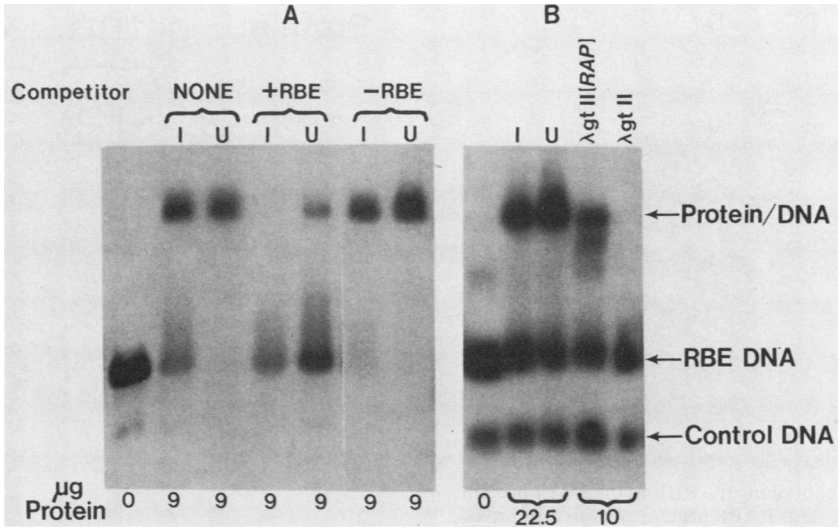

FIG. 7. Protein-DNA complexes formed in crude extracts with labeled DNA containing a RAP1-binding site. (A) Complexes formed with labeled DNA 235 base pairs in length extending from −306 to −527 (and including *Hin*dIII and *Kpn*I linkers and pBR322 sequence from *Hin*dIII to *Eco*RI). Total protein was 9 μg per lane, and the extracts were made from cells that were either induced with MMS (I) or uninduced (U). In some cases, cold competitor DNA either containing (+RBE; same fragment as labeled DNA) or lacking (−RBE; −409 to −527 plus linkers and pBR322 sequence from *Hin*dIII to *Eco*RI) RAP1-binding sites was added in 100-fold molar excess. (B) Protein-DNA complexes formed in induced and uninduced extracts next to the complex formed in extracts from an *E. coli* λgt11 lysogen carrying the *RAP1* gene [λgt11 (*RAP*)]. The absence of a protein-DNA complex in λgt11 lysates without the *RAP1* gene is also shown. The control DNA is a segment of the *RNR2* promoter extending from −335 to −182.

both MMS-induced and uninduced extracts bound to the *RNR2* fragment. The protein bound to the RBE-containing DNA is identified as RAP1 for the following reasons. (i) Binding activity was overproduced in extracts made from BJ2168(pGALRAP), which overproduced the RAP1 protein in the presence of galactose (Fig. 8). The increase in bound *RNR2* DNA upon RAP1 induction with galactose appeared to be more than the 10-fold overproduction of the protein, but binding may not have been linear with increasing protein concentration (Fig. 9). (ii) The *RNR2* DNA-binding protein was present in a λgt11 lysogen carrying the *RAP1* gene (in an orientation opposite that of *lacZ*) but was absent in a λgt11 lysogen lacking the *RAP1* gene or in a lysogen carrying the gene in the reverse orientation, neither of which made the RAP1 protein (Fig. 8). (iii) The protein was 95 to 105 kilodaltons (kDa) in size relative to RNA polymerase subunit markers (Fig. 9), consistent with the 92.5-kDa size of the RAP1 protein predicted by the DNA sequence of the *RAP1* gene (47). The λgt11 lysates also were probed with a labeled fragment containing *RNR2* sequences upstream of the RBE. An *E. coli* protein bound to this fragment and to the RBE-containing fragment, suggesting that this protein may bind DNA nonspecifically (Fig. 9). Additional proteins binding the RBE-containing fragment that appeared only in the λgt11(*RAP*) lysate lane may have been proteolytic fragments of RAP1. Figure 7B shows further evidence of RAP1 proteolysis; some of the complexes formed in λgt11(*RAP*) extracts migrated faster than the putative RAP1 complex. The RAP1 protein was the same size in λgt11(*RAP*) lysates, in extracts of yeast carrying the *GAL1-RAP1* fusion grown in either glucose or galactose, and in extracts of wild-type yeast cells grown in rich medium with or without MMS (Fig. 8).

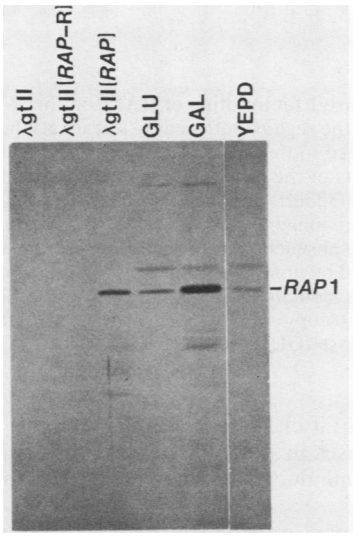

FIG. 8. Immunoblot identifying the RAP1 protein. Yeast crude extracts were prepared either from cells carrying *GAL1-RAP1* fusion sequences and grown in the presence of minimal medium and glucose (GLU) or galactose (GAL) or from cells with a single endogenous copy of *RAP1* grown in rich medium (YEPD). *E. coli* lysates of a λgt11 lysogen or a λgt11 lysogen carrying the *RAP1* gene in the opposite orientation as *lacZ* [λgt11(*RAP*)] or a λgt11 lysogen with *RAP1* in the reverse orientation [i.e., in the same orientation as *lacZ*; λgt11(*RAP*-R)] are also shown. RAP1 protein was detected by RAP1 polyclonal antibodies, and each lane contained 25 μg of total protein.

Protection of the RBE from DNase I cleavage by the RAP1 protein. Figure 10 shows protection of the RBE from DNase I digestion that can be ascribed to RAP1 binding. Extracts made from BJ2168(pGALRAP) cells grown in either glucose or galactose protected the RBE, but a dose curve of increasing protein showed that extracts made from cells grown in galactose protected with less protein (0.5 to 2 μg) than did extracts made from cells grown in glucose (11 μg). This

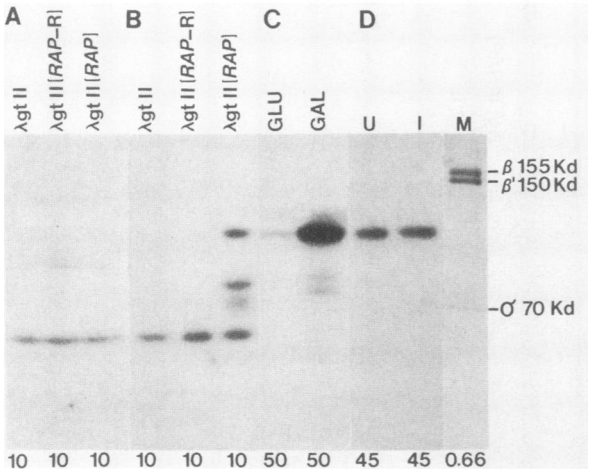

FIG. 9. Binding of DNA containing the RBE to the RAP1 protein in a Southwestern analysis. The RAP1 protein migrated approximately halfway between the β subunit and the σ subunit of *E. coli* RNA polymerase (identified by antibodies to RNA polymerase), shown in lane M as size markers. Lanes were incubated with an end-labeled fragment of the *RNR2* promoter not carrying *RAP1* sequences (−409 to −527) (A) or with labeled DNA carrying RAP1-binding sequences (−306 to −527) (B through D). Amounts of protein assayed (in micrograms) are shown at the bottom. (A and B) *E. coli* lysates from a λgt11 lysogen containing no insert DNA, a λgt11 lysogen carrying the *RAP1* gene in the same orientation as *lacZ* [λgt11 (*RAP*-R)], and a λgt11 lysogen carrying the *RAP1* gene in the opposite orientation [λgt11(*RAP*)]. (C and D) Labeled fragment containing the RBE bound to the RAP1 protein in yeast crude extracts. Extracts in panel C were prepared from cells carrying the *RAP1* gene fused to the *GAL1* promoter that were grown in the presence of glucose (GLU) or galactose (GAL). Extracts in panel D were prepared from cells lacking this construct that were grown in YEPD and induced by MMS (I) or uninduced (U). Exposures of panels A through D were different, so the amount of RAP1 cannot be quantitatively compared in these panels. However, direct comparisons can be made within experiments; e.g., compare RAP1 from cells grown in the presence of glucose versus galactose in panel C.

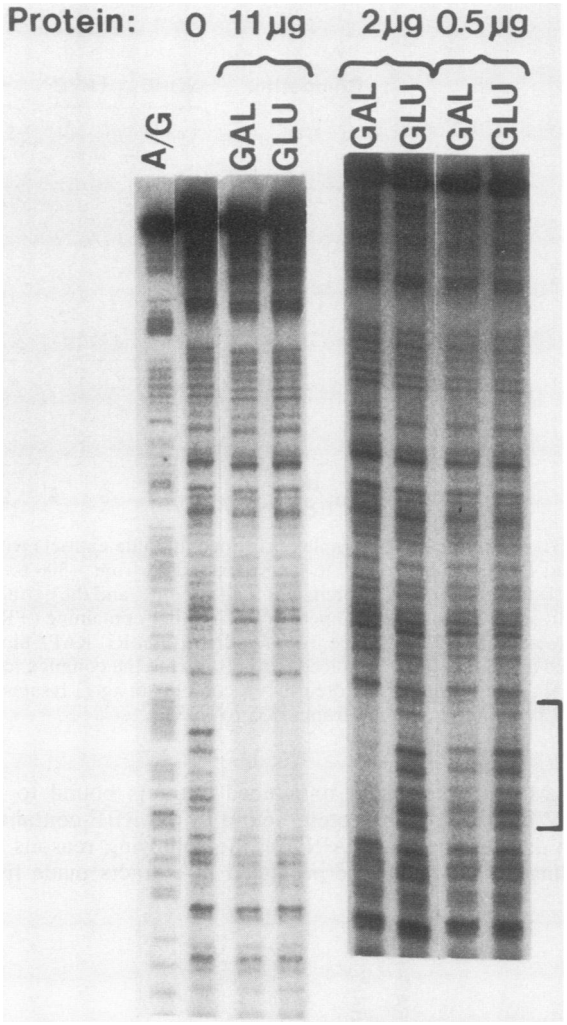

FIG. 10. DNase I footprinting of RAP1 on the *RNR2* promoter. RAP1 protected the region indicated by a bracket, the boundaries of which were related to the known sequence by comparison with the cleavage products of the same labeled DNA fragment generated by the Maxam and Gilbert A-G reaction shown in the leftmost lane. Labeled DNA in unmarked lane shows the pattern of DNase I cleavage in the absence of protein. The other lanes show the protection caused by limiting amounts of crude extract (0.5 to 11 μg) made from cells containing sequences with the *GAL1* promoter fused to an extra copy of the *RAP1* gene that were grown in the presence of glucose (GLU) or galactose (GAL).

result is consistent with the 10-fold overproduction of RAP1 in BJ2168(pGALRAP) cells grown in galactose. Protection on the bottom strand extended from −338 to −358, a 21-base-pair region that directly covers the RBE (which extends from −344 to −356), one of the sequences identified as a potential RAP1-binding site by its homology to the consensus for high-affinity binding of RAP1 (3). The other sequence that showed homology to RAP1-binding sites was not protected from DNase I cleavage by as much as 72 μg of protein from BJ2168(pGALRAP) extracts made from cells grown in galactose. The top strand showed a protection pattern similar to that seen on the bottom strand: a region protected around the RBE and no protection at the other potential RAP1 binding site (data not shown).

DISCUSSION

The *RNR2* gene is induced by various DNA-damaging agents. Induction of the *RNR2* transcript by at least one agent, MMS, does not require new protein synthesis. MMS induces a *RNR2-lacZ* fusion 18-fold, which is comparable to the MMS-induced 17- and 8-fold increases in the *RNR2* mRNA and protein, respectively (24). Deletions in the upstream region of *RNR2* alter the induction ratio of the *RNR2-lacZ* fusion, suggesting that the increases in *RNR2* mRNA are at least partially regulated by changes in the rate of transcription; if changes in mRNA stability were responsible for increases in steady-state levels of mRNA, the ratio of induced to uninduced activity should remain the same for every deletion.

RNR2 deletions, ranging from −528 to the mapped transcriptional start site at +1, identify a fragment of about 200 base pairs that causes a *CYC1-lacZ* fusion to respond to MMS when this segment is attached upstream of the *CYC1* transcriptional start site. This region of the *RNR2* promoter contains negative and positive regulatory sequences, just as does the promoter that regulates the expression of ribonucleotide reductase in *E. coli* (57). The URS is flanked by two positive activation sites, one or the other of which is required for *RNR2* expression when the repression sequence is deleted. The downstream positive activation sequence

contains an RBE that binds the RAP1 protein in vitro, as shown by gel electrophoresis retardation, Southwestern analysis, and footprinting.

The behavior of the deletions suggests that at least part of the regulation of *RNR2* may occur through a relief of repression, caused by MMS, that then allows more efficient action of two positive activators. Interestingly, the promoters of two other genes, *ENO1* (7) and *PYK* (39), contain a RAP1-binding site, another unrelated activation site, and a URS that are required for the regulation of each gene; furthermore, glucose-induced expression is thought to occur through relief of repression in each case. For *RNR2*, MMS could directly effect derepression by deactivating a repressor protein. Alternatively, repression might result from a complex protein-DNA structure that is particularly dependent on sequences in the URS and perhaps somewhat dependent on other unidentified sequences; then MMS might directly or indirectly disrupt any facet of the structure.

The protein-DNA complex formed by a segment of the *RNR2* promoter with proteins of uninduced and induced yeast extracts requires the RBE, because an otherwise identical DNA fragment without the RBE does not compete for binding (Fig. 7A). Furthermore, a protein-DNA complex is formed in lysates of λgt11(*RAP*) (and not in lysates of λgt11) that migrates identically to complexes formed in yeast extracts (Fig. 7B). These data suggest that only the RAP1 protein bound to the RBE is responsible for the altered mobility of the RBE-containing fragment in both λgt11 (*RAP*) lysates and yeast extracts, because no additional protein should be common to both. It is possible, although perhaps not likely, that other proteins from either extract which do not affect the mobility of the complex are bound.

We found no reproducible difference in RAP1-binding activity in MMS-induced and uninduced extracts, suggesting that MMS does not change the amount of RAP1-binding activity. This result is not surprising, since RAP1 binds the regulatory regions of many genes. It is perhaps most likely that RAP1 is a general activator and that a different factor or factors respond to MMS. As suggested by the behavior of deletions, this regulatory protein could be a repressor or another protein that prevents repressor action. However, RAP1 still might respond directly to MMS even if it is always bound; induction then could involve a modification of prebound RAP1 that allows transcriptional activation, as in the case of the yeast heat shock factor (26). It is also possible that RAP1 is not bound to RBE DNA in vivo in the uninduced state because a particular chromatin structure restricts binding; in this case, induction could involve a rearrangement that allows RAP1 to bind.

RAP1 is an abundant protein that binds to the promoters of many genes, including the ribosomal genes *TEF1*, *TEF2*, and *RP51A* (23), *MATα*, and the genes for a variety of glycolytic enzymes and other proteins involved in cell growth (4). RAP1 also binds sites in telomeres (1) and a site in the E regions of HMR and HML (48). (RAP1 is an acronym for repressor-activator protein, since it appears to be involved in silencer function at HMR E and HML E and in transcriptional activation.) The prevalence of RAP1-binding sites suggests that RAP1 may act as a general DNA-binding protein like factor Y, a protein thought to modify chromatin structure to allow regulatory proteins to bind (10). The RAP1 protein (previously called SBF-E), whose gene was cloned by Shore and Nasmyth (47), also has been named TUF and GRF1 in different systems; the inferred identity of these proteins is based on the similarity between sequences to which they bind and on their similar abundances and sizes. Buchman et al. (3) have shown that *TEF2*, HML E, and telomere sequences compete with *MATα* sequences for binding to an abundant factor in yeast extracts, and Shore and Nasmyth (47) have shown that *TEF2*, *RP51*, and HML E sequences compete for binding to the RAP1 protein.

We detected RAP1 as a protein of 95 to 105 kDa according to its mobility in SDS gels, which was identical whether the source was normal yeast extracts, yeast extracts that overproduce RAP1, or *E. coli* λgt11(*RAP*) lysates. Shore and Nasmyth identify RAP1 as a ~120-kDa protein (47), a difference that could reflect the molecular size markers used. We detected a faint band of higher molecular size recognized by the RAP1 antibody that could be an unproteolyzed form of RAP1; this protein is minor or nonexistent in extracts made in a different way (rapid disruption of cells with base and β-mercaptoethanol, followed by immediate loading on polyacrylamide-SDS; data not shown). We detected no specific DNA-binding activity of the larger protein, suggesting either that it is not related to RAP1 and represents a spurious cross-reaction to RAP1 antibodies or that it is a modified form that does not bind the RBE. Since λgt11(*RAP*) extracts contain RAP1 of normal size, we presume that the polypeptide is initiated at or near its normal site and is not a fusion to β-galactosidase. We do not know why the *RAP1* gene is expressed in only one orientation in λgt11 clones nor what promoter is used.

DNase I footprinting shows that RAP1 protects the RBE as expected; the binding species is RAP1, because the binding activity is overproduced in BJ2168(pGALRAP) cells that overproduce RAP1. The other potential RAP1-binding site that more loosely matches the proposed consensus high-affinity binding site (5′-A/GA/CACCCANNCAT/CT/C-3′) suggested by Buchman et al. (3) is not protected from DNase I digestion when even 100 times the amount of protein required to protect the RBE is used. This result is consistent with its poorer match (11 of 13) than that of the RBE (12 of 13) to the high-affinity consensus sequence and with the fact that it is different in 5 of 13 base pairs from the RBE (compare the RBE and the other potential RAP1-binding site underlined in Fig. 4 with the consensus sequence above). It is possible that the second site binds RAP1 in vivo or that it binds a much higher concentration of RAP1 in vitro. However, deletions in this region do not suggest that the second sequence functions in the regulation of *RNR2* expression.

There is no genetic evidence to reveal the effect of loss of RAP1 function on *RNR2* expression. However, we have shown that overproduction of RAP1 has no effect on *RNR2* expression in the presence or absence of MMS (H. K. Hurd and J. W. Roberts, unpublished data), as might be expected, since RAP1 is already an abundant protein.

We identified several DNA-damaging agents that induce the *RNR2* promoter and some that do not. Active agents cause a variety of different DNA lesions, including alkylation damage (MMS), thymine dimers (UV light), breaks in DNA (BLE and HU), and possibly damage resulting from the interruption of DNA synthesis (HU). Because chemicals that damage DNA also inhibit DNA replication, and some that inhibit replication also may lead to DNA damage, it is unclear whether DNA damage per se or the interruption of DNA synthesis is the event that leads to *RNR2* induction. In *E. coli*, ribonucleotide reductase is induced in temperature-sensitive mutants that stop DNA synthesis at the nonpermissive temperature, as well as by agents that damage DNA (11, 12, 18). However, these experiments are complicated in

yeast cells because ribonucleotide reductase activity is cell cycle regulated (33); it is only expressed in S phase when DNA replication occurs. Therefore, if inhibition of DNA synthesis allows cells to collect in S phase, which normally lasts one-third to one-fourth of the cell cycle, then arrest alone may increase the apparent amount of ribonucleotide reductase. It is consistent with this view that *RNR2-lacZ* expression increases fourfold when a *cdc8* mutant (thymidylate kinase [45]) is arrested in S phase by growth at the nonpermissive temperature. In contrast, *RNR2-lacZ* expression does not increase in a *cdc17* mutant (51) that arrests at the border between G2 and M phase at the nonpermissive temperature (Hurd and Roberts, unpublished data). Thus, DNA-damaging agents or DNA synthesis inhibitors could induce *RNR2* expression because they cause cell cycle arrest at a time when *RNR2* is transcriptionally active. However, the fact that many agents, including MMS, induce the *RNR2* gene more than simple cell cycle arrest does suggests there may exist regulation, independent of cell cycle control, that responds to a signal generated by DNA damage. One possibility is that variations in nucleotide pools provide this signal. However, no simple relationship between the induction of ribonucleotide reductase and nucleotide availability has been shown; for example, excess thymidylate and thymidylate starvation induce ribonucleotide reductase activity in yeast cells equally well (30).

The induction of *RNR2* mRNA by MMS is not affected detectably by CH and thus is independent of protein synthesis (Fig. 2). In contrast, *E. coli* ribonucleotide reductase requires protein synthesis for induction (19). Growth of yeast cells in CH arrests cells in interphase, including G1, S, and G2, when protein synthesis occurs. If *RNR2* mRNA induction were dependent on S-phase arrest, growth in CH should decrease mRNA induction because CH prevents cells from entering S phase from G1 (21). However, since *RNR2* induction is similar in cells grown with and without CH, the mechanism of induction is probably at least partially independent of cell cycle control.

Although induction of *RNR2* appears to occur outside of S phase, some part of the increase in *RNR2* expression caused by DNA-damaging agents could result from cell cycle arrest. Thus, the 15-fold induction of the wild-type *RNR2* gene (deletion −528) by MMS might be a composite of a gene-specific effect mediated by the URS and the more general effect of stopping cells at the S/G2 border (as MMS does [28]); the three- to fourfold induction that remains after the URS is deleted might reflect only the component of induction due to arrest of growth in S/G2. Weinert and Hartwell (59) described a mutation, *rad9*, that prevents cell cycle arrest after DNA damage; thus, this mutant should reduce any component of *RNR2* induction due to arrest at the S/G2 border of the cell cycle. It is consistent with the interpretation discussed above that *RNR2* induction by MMS is only 25 to 50% of the wild-type level in *rad9* cells (Hurd and Roberts, unpublished data), although the relationship of the URS to the *rad9* effect has not been tested.

The relationship of induction by DNA damage to the cell cycle has been considered for other yeast genes. Johnston et al. found that induction of the cell-cycle-regulated *POL1* transcript in response to UV occurs normally in cells arrested in G1, at a time well before *POL1* mRNA is made, which led them to conclude that induction is independent of cell cycle controls (27). In contrast, Kupiec and Simchen (29) have shown that the temperature-sensitive *cdc40* mutant, which arrests in S/G2 at the nonpermissive temperature, causes an induction of the cell-cycle-regulated *RAD6* gene that is equal to the induction of *RAD6* caused by MMS and HU, which arrest cells at the same time in the cell cycle (28). As was suggested for *RAD6* induction by MMS, yeast cells may have a cellular mechanism to specifically arrest cells with damaged DNA at a point in the cell cycle when repair enzymes are synthesized rather than inducing repair enzymes in response to a DNA damage signal as *E. coli* does (32).

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