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Identification of the DNA Damage-Responsive Element of *RNR2* and Evidence that Four Distinct Cellular Factors Bind It

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The *RNR2* gene encodes the small subunit of ribonucleotide reductase, the enzyme that catalyzes the first step in the pathway for the production of the deoxyribonucleotides needed for DNA synthesis. Transcription of this gene is induced approximately 20-fold in response to environmental stimuli that damage DNA or block DNA replication. Deletion and subcloning analysis identified two, and possibly three, upstream activating sequences (UAS) and one repressing (URS) element in the *RNR2* regulatory region. A 42-base-pair (bp) fragment from this region was found to be necessary for proper regulation of *RNR2* and to be capable of conferring DNA damage inducibility upon a heterologous promoter. This fragment contained both positively and negatively acting sequences. Four DNA-binding factors interacted with the *RNR2* regulatory region. One factor was identified as the GRF1 protein, the product of the *RAP1* gene. GRF1 bound to the UAS2 element of *RNR2*, which was found to be directly adjacent to the 42-bp fragment. UAS2 activity was repressed by the 42-bp fragment. Three other factors bound to the 42-bp fragment; one of these factors, RRF3, had a second binding site in the *RNR2* promoter. These factors are likely to mediate the response of *RNR2* to DNA damage.

The replication of eucaryotic chromosomes is a highly organized and regulated process. One level of this organization results from the restriction of replication to a defined period of the cell cycle, S phase. This is accomplished, at least in part, by the temporal modulation of expression of gene products needed specifically in S phase. In *Saccharomyces cerevisiae*, not only are genes encoding the enzymatic machinery for DNA synthesis cell cycle regulated (*POL1* [DNA polymerase I] [13] and *CDC9* [DNA ligase] [2, 19, 24]), but so are many of the enzymatic activities involved in the production of the deoxyribonucleoside triphosphate precursors needed for DNA synthesis (*CDC8* [thymidylate kinase] [23], *CDC21* [thymidylate synthase] [23], and ribonucleotide reductase [16]).

Multiple forms of regulation are often interwoven into the control of cell-cycle-regulated genes because although these genes are required primarily in one period of the cell cycle, circumstances can arise in which their expression is needed outside of the period in which they are normally expressed. For example, repair of certain types of DNA damage requires the ability to synthesize DNA. If repair is to proceed outside of S phase, then the cell must encode the capacity to synthesize DNA in other phases of the cell cycle. In fact, several genes with cell-cycle-regulated activities involved in DNA synthesis are inducible by DNA damage (*POL1* [13]), *CDC9* [2, 19], *CDC8* [8], *RNR2* [8], and *RNR1* and *RNR3* [S. J. Elledge and R. W. Davis, manuscript in preparation]). The induction of these genes in response to the stress of DNA damage is thought to produce a metabolic state that facilitates DNA replicational repair processes (8a). To explore the ability of cells to sense and respond to DNA damage, we have examined the regulation of the genes encoding the enzyme ribonucleotide reductase.

Ribonucleotide reductase catalyzes the first step in the pathway for the production of the deoxyribonucleotides needed for DNA synthesis. It is an enzyme of structure $\alpha_2\beta_2$. In yeast cells, the small subunit is encoded by *RNR2* (8, 11), and the large subunit is encoded by two homologous genes, *RNR1* and *RNR3* (Elledge and Davis, in preparation). The activity of the enzyme fluctuates in the cell cycle, with a maximum in S phase (16). The mechanism responsible for this regulation is not known.

The amount of ribonucleotide reductase is also increased under conditions of nucleotide depletion (15). This increase in activity is due to increased transcription of the genes encoding ribonucleotide reductase. The regulation of *RNR2* has been studied most extensively (8, 8a, 11). It is induced in response to a wide variety of agents that either damage DNA directly through chemical modification or induce stress by blocking DNA synthesis (8a). The induction of *RNR2* mRNA can occur in G1 or S phase and is not blocked by the presence of cycloheximide (8a). Cells bearing a mutation in the structural gene of *RNR2*, *rnr2-314*, are partially constitutive for the DNA damage response. However, this is likely to be an indirect autoregulatory circuit because overproduction of *RNR2* has no regulatory phenotype. Other cellular mutations can alter the sensitivity to inducing agents, but none are known to block induction.

We have undertaken a detailed analysis of the *RNR2* regulatory region to identify the *cis*-acting sequences involved in mediating the transcriptional response to the stress of DNA damage. This analysis has revealed the complex nature of the *RNR2* regulatory region. Furthermore, we have identified a 42-base-pair (bp) region that contains both an upstream repressor sequence (URS) and an upstream activator sequence (UAS) and is capable of conferring damage inducibility upon a heterologous promoter.

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. Selective medium was minimal medium supplemented with various amino acids and bases, prepared as described by Sherman et al. (20), as was yeast extract-peptone-dextrose (YPD) medium. Hydroxyurea

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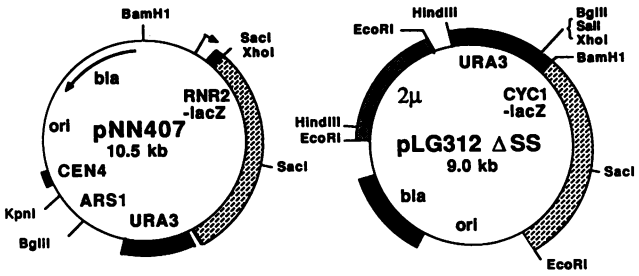


FIG. 1. Structures of the plasmids used to analyze the regulatory region of *RNR2*. pNN407 is a centromeric vector containing approximately 960 bp of *RNR2* DNA upstream of the *Nsi*I site in codon 12 of the *RNR2* coding region, where it is fused to the *Pst*I site of a modified *lacZ* gene as described in Materials and Methods. The stippled region preceding the *Sac*I site represents the coding region of *RNR2*. The decorated region clockwise from the *Sac*I site represents the *lacZ* gene. The *RNR2-lacZ* gene fusion results in a protein fusion between the respective polypeptides. pLG312 ΔSS is a 2μm-based UAS probe vector. It contains a *CYC1* gene with a promoter in which a deletion has removed all UAS sequences. The coding region of *CYC1* is represented by the stippled region directly preceding the *Bam*HI site. The *CYC1* gene is fused in frame to a derivative of the *lacZ* gene. This gene fusion results in the production of an enzymatically active CYC1–β-galactosidase protein fusion.

(HU) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Strains and plasmids. The host *S. cerevisiae* strain used was LN114 (8). DNA was introduced into LN114 by the method of Ito et al. (12). The *Escherichia coli* strain used as a host for constructions and plasmid amplification was JM107 (25). pLG312 ΔSS and was a gift of A. Mitchell (Columbia University). pLG312 ΔSS is a derivative of pLG312 (9, 10) with a *Bgl*II linker inserted between the *Sal*I and *Sma*I sites, thus removing the UAS of *CYC1*. pNN407 (Fig. 1) was derived from pNN405 (8a) by inserting a *Bam*HI linker into *Eco*RI-cleaved, T4 polymerase-flushed pNN405.

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 and Ptashne (10). Yeast strains were grown overnight in selective minimal medium for all experiments described. They were diluted into fresh YPD medium and grown to mid-log phase (optical density of 0.3), and HU was added to a final concentration of 100 mM. After 4 h of treatment, 3 ml of cells was pelleted, washed, and suspended in 1.0 ml of water. A 0.5-ml sample of cell suspension was added to 0.5 ml of 0.37% formaldehyde solution for later determination of optical density at 600 nm. The remaining 0.5 ml was added to 0.5 ml of Z buffer (18) 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 4-mg/ml ONPG. The rest of the assay and unit calculations were as described by Miller (18). This regimen produced activity measurements that were reproducible within 20%. Assays were performed in duplicate and averaged.

5′ deletion series. The *Cla*I-*Xho*I fragment of pNN403 (8a) containing the *RNR2* regulatory region through the translational start site (*Cla*I-*Nsi*I), was cloned into *Cla*I-*Xho*I-cleaved pBS KS+ (Stratagene, San Diego, Calif.) to produce pSE621. A series of nested deletions was made in this plasmid as described previously (8). The common end of the deletions was a *Sma*I site in the polylinker of pBS KS+. This deletion series was excised from this vector by cleavage with *Bam*HI and *Xho*I and ligated into *Bam*HI-*Xho*I-cleaved pNN407 to produce the series used in Fig. 2. Selective deletions were sequenced as described previously (8). Deletions to particular restriction sites were made by cleaving the plasmid with the enzyme of interest and *Sma*I, making flush with T4 polymerase, and ligating shut.

3′ deletions and subclones. Fragments used to determine the effects of 3′ deletions were usually first cloned into polylinker vectors and then excised with the proper restriction enzymes for insertion in pLG312 ΔSS. For all constructions mentioned, the designation (blunt) after a restriction enzyme name indicates that after cutting with that enzyme, that particular site was made blunt by treatment with T4 polymerase and all four deoxyribonucleoside triphosphates. For Fig. 3, fragment 1 was isolated as a *Hin*dIII-*Xho*II (blunt) fragment and cloned into *Hin*dIII-*Hin*cII-cleaved pBS KS+ to create pSE747. The fragment was then excised in its entirety on a *Bam*HI-*Xho*I fragment and ligated into *Bgl*II-*Xho*I-cleaved pLG312 ΔSS to produce subclones in the positive orientation. To produce clones in the negative orientation, a three-way ligation was performed by using the above *Bam*HI-*Xho*I fragment, the small *Bgl*II-*Sac*I fragment of pLG312 ΔSS containing the 5′ end of *CYC1-lacZ*, and the large *Xho*I-*Sac*I fragment of pLG312 ΔSS containing the origin. Fragments 2 to 5 were cloned in the same manner by using different starting fragments. The starting fragments were as follows: for fragment 2, *Hin*dIII-*Dde*I(blunt); for fragment 3, *Hin*dIII-*Rsa*I(blunt); and for fragment 5, *Hin*dIII-*Sty*I(blunt). Fragment 4 was slightly different in that its 3′ endpoint was the artificially induced *Not*I site shown in Fig. 4. First, the *Bgl*II-*Not*I D1 oligonucleotide (Table 1) was cloned into *Bgl*II-*Not*I-cleaved pSE387 [pSE387 is pIC20H (18) with a *Not*I linker inserted at the *Eco*RV site] to make pSE863 (Davis strain collection designation pNN411). This creates the following order of restriction sites: *Bam*HI *Not*I(3′ D1) *Sty*I *Bgl*II(5′ D1) *Xho*I. The *Bam*HI-*Sty*I fragment from pSE747 was introduced into *Bgl*II-*Sty*I-cut pSE863 to produce pSE877. The *Bam*HI-*Xho*I fragment was excised from this plasmid and ligated into pLG312 ΔSS as described above.

5′ deletion plasmids generated for Fig. 2, which placed a *Bam*HI site at the 5′ end of the deletions, were used as a source of restriction fragments for fragments 6, 7, and 9 of Fig. 3. Fragment 6 was excised from the Δ24 deletion as a *Bam*HI-*Dde*I(blunt) fragment and ligated into *Bgl*II-*Xho*I (blunt) pLG312 ΔSS to produce the positive-orientation clone. To produce clones in the negative orientation, a three-way ligation was performed by using the abovementioned *Bam*HI-*Dde*I(blunt) fragment, the small *Bgl*II-*Sac*I fragment of pLG312 ΔSS containing the 5′ end of *CYC1-lacZ*, and the large *Xho*I(blunt)-*Sac*I fragment of pLG312 ΔSS containing the origin. In a similar manner, the Δ30 deletion provided fragment 7 as a *Bam*HI-*Dde*I(blunt) fragment for cloning, Δ24 provided fragment 9 as a *Bam*HI-*Rsa*I fragment for cloning, and the resulting plasmid was named pNN410. Fragment 8 was produced as a *Bgl*II-*Rsa*I fragment for cloning by cleaving pSE621 with *Hpa*II, ligating on a *Bgl*II linker, cleaving with *Bgl*II and *Rsa*I, and isolating the fragment for subcloning as above.

The oligonucleotides used are shown in Table 1. Clones shown in Fig. 4 were created by ligating the appropriate oligonucleotide with *Bgl*II-*Xho*I ends, or pairs of oligonucleotides, as was the case for fragment D1,2, into pLG312 ΔSS.

TABLE 1. Oligonucleotides used

Oligonucleotide	Length (bp)	Sequence[a]
D1	43	GATCTACCACACCCACGCGCGATCGCCATGGCAACGAGGTCGC ATGGTGTGGGTGCGCGCTAGCGGTACCGTTGCTCCAGCGCCGG
D2	42	GGCCGCCCCACACCCAGACCTCCCTGCGAGCGGGCATGGGTC CGGGGTGTGGGTCTGGAGGGACGCTCGCCCGTACCCAGAGCT
Dxb	47	GATCTACCACACCCACGCGCGATCGCCATGGCAACGAGGTCGCACAC ATGGTGTGGGTGCGCGCTAGCGGTACCGTTGCTCCAGCGTGTGAGCT
FII	38	GATCTGTCGACAGACCTCCCTGCGAGAGGGCATGGGTC ACAGCTGTCTGGAGGGACGCTCTCCCGTACCCAGAGCT
α UAS	31	GATCCATCCCAAACAAAACCCAGACATCATG GTAGGGTTTGTTTTGGGTCTGTAGTACTTAA
ENO1	38	GATCCGAGCTTCCACTAGGATAGCACCCAAACACCTGG GCTCGAAGGTGATCCTATCGTGGGTTTGTGGAGGTTAA
ENO1mtA	38	GATCCGAGCTTCCACTAGGATAGCACaCAAACACCTGG GCTCGAAGGTGATCCTATCGTGtGTTTGTGGAGGTTAA
EII	31	GATCTTATATTGCAAAAACCCATCAACCTTG AATATAACGTTTTTGGGTAGTTGGAACTTAA
EIImtA	31	GATCTTATATTGCAAAAACaCATCAACCTTG AATATAACGTTTTTGtGTAGTTGGAACTTAA

[a] Both strands of each oligonucleotide are shown, with the top strand listed 5′ to 3′ (left to right). Base pair changes in the mutant oligonucleotides ENO1mtA and EIImtA are shown as lowercase letters. The last five oligonucleotides listed were gifts from A. Buchman.

The D1 subclone was made by deleting between the *Not*I and *Xho*I sites of the D1,2 subclone. The D2 subclone was created by deleting between the *Bgl*II and *Not*I sites of the D1,2 subclone. The 44-bp *Mnl*I fragment was ligated into the *Sma*I site of pICEM 19+(18), sequenced to verify its orientation, excised as a *Bam*HI-*Sal*I fragment, and cloned in both orientations into pLG312 ΔSS. The Sty-Rsa subclone was created by taking the pBS KS+ subclone of fragment 3 (see Fig. 3) and deleting between the *Sty*I and *Eco*RV sites. This deletion fragment could then be excised as a *Bam*HI-*Xho*I fragment introduced into pLG312 ΔSS.

Constructs for Fig. 8 were primarily derived from the D1 subclone pNN411 (see above). The 4-bp insertion mutation in D1 was created by cleaving pSE863 with *Sty*I and filling in with the large fragment of DNA polymerase I to create pNN412. Insertion of the α UAS fragment into pSE387, pNN411, and pNN412 was achieved by cleaving each of these molecules with *Bam*HI and *Eco*RI and ligating in the *Bam*HI-*Eco*RI α UAS oligonucleotide described in Table 1. The fragments of interest were excised from these plasmids as *Bam*HI-*Xho*I fragments and introduced into pLG312 ΔSS in both orientations as described above. The Dxb oligonucleotide was cloned into the *Bgl*II-*Sal*I polylinker sites of plasmid pMTL21 and excised as a *Xho*I-*Bam*HI fragment; it was then labeled as a probe for gel shifts or used directly as a competitor. The sequence surrounding the Dxb fragment is (*Xho*I) CTCGAG-*Bgl*II-Dxb-(*Xho*I-*Sal*I) CTCGACGTCAT ATGGATCC (*Bam*HI). Sequences of the sites in parentheses are underlined.

Whole-cell and nuclear extracts. Yeast whole-cell extracts were prepared as described previously (5, 6) by glass bead disruption of cells in buffer A (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 10% glycerol, 1 nM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 0.6 μM leupeptin, 5 μg of antipain per ml, 50 mM KCl) containing 0.3 M ammonium sulfate. Protein concentrations were measured by the method of Bradford (3). Nuclear extracts were a gift of N. Lue (Stanford University). All extract were prepared from strain BJ926 (4).

Protein-DNA binding assays. Generally, gel electrophoresis mobility shift assays were performed as described by Buchman et al. (5). Binding reactions were done in 20 μl of buffer A containing 5 to 10 fmol of ^{32}P-labeled probe DNA, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), and various amounts of yeast protein and competitor DNA. After incubation at room temperature for 10 min, reactions were electrophoresed in a 4% polyacrylamide gel. These gels were then dried and autoradiographed.

RESULTS

***lacZ* fusions used in analysis of the *RNR2* regulatory region.** Two plasmids were used to analyze the regulatory region of *RNR2* (Fig. 1). pNN407, a derivative of a previously characterized *RNR2-lacZ* fusion plasmid, pNN403 (Elledge and Davis, submitted), was used to construct and characterize a series of 5′ deletion mutations in the *RNR2* regulatory region. This derivative, pNN407, contains the *lacZ* gene fused to the amino terminus of *RNR2* (at amino acid 12) plus about 1,000 bp of DNA upstream of the *RNR2* transcriptional start. *S. cerevisiae* cells containing this plasmid synthesize low levels of β-galactosidase constitutively and 10-fold higher levels when treated with agents that damage DNA or block DNA replication (8a).

pLG312 ΔSS is a derivative of pLG312, a 2μm-based plasmid containing a *CYC1-lacZ* fusion (9, 10). pLG312 ΔSS was created by deletion of the *Sma*I-*Sal*I fragment from pLG312 containing the *CYC1* UAS region and replacing it with a *Bgl*II linker (A. Mitchell, personal communication). *S. cerevisiae* cells containing this plasmid do not synthesize detectable levels of β-galactosidase. Thus, pLG312 ΔSS was used to analyze 3′ deletions of the *RNR2* regulatory region as well as 5′,3′ deletion mutations.

5′ deletion mutations define several regions important for *RNR2* expression. A series of 20 5′ deletions was created in the *RNR2* regulatory region (Fig. 2A) and introduced into pNN407 as described in Materials and Methods. The effects of these deletions on β-galactosidase synthesis in strains bearing these pNN407 derivatives were determined under normal conditions and in the presence of the *RNR2* inducer HU (Fig. 2). The numbering system used arbitrarily uses the *Nsi*I site of *RNR2*, which is the location of the fusion to *lacZ*, as 0.

The behavior of these deletions suggested the presence of a URS between −489(Δ24) and −460. Deletion of this

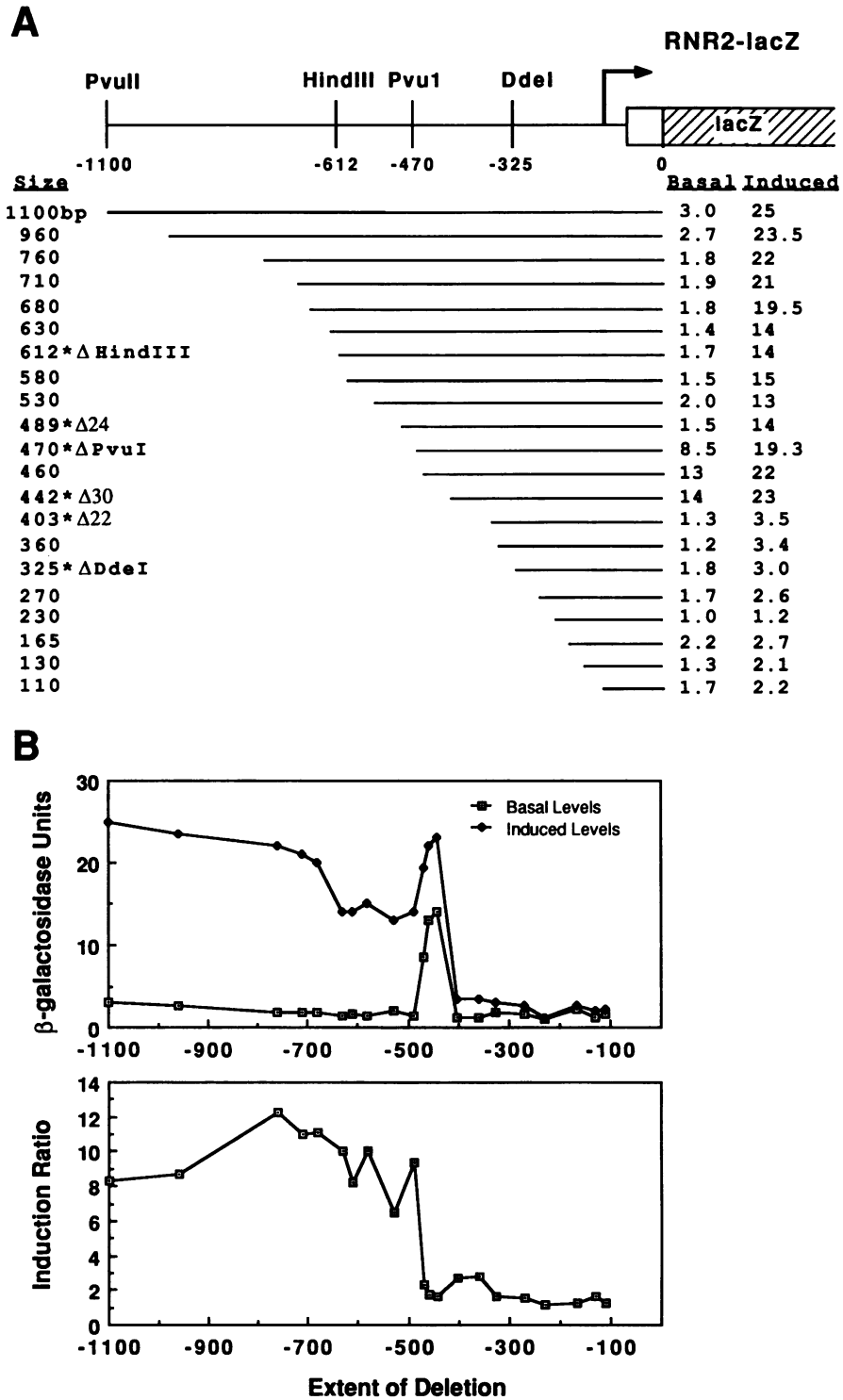
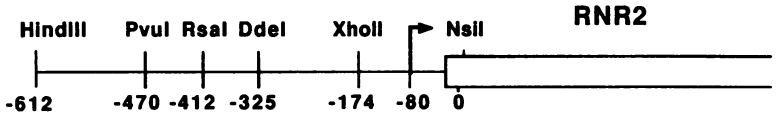


FIG. 2. Effects of 5′ deletions on *RNR2* promoter activity. (A) Restriction map of the *RNR2* regulatory region present on pNN407. The *NsiI* site at the *RNR2-lacZ* fusion point was arbitrarily chosen as the zero coordinate. The lines below indicate the amount of DNA remaining in each deletion derivative. The length of *RNR2* DNA in each derivative is listed on the left. Names of selected deletions are listed adjacent to their sizes. Numbers on the right represent the β-galactosidase activity in strains bearing these constructs after 4 h in the absence (basal) or presence (induced) of 100 mM HU. *, Deletions whose endpoints were sequenced to determine their precise boundaries. (B) Plot of the values obtained in panel A. The *x* axis is the extent of deletion and is colinear with the restriction map of the *RNR2* regulatory region at the top of part A. The induction ratio used in the lower plot was calculated by taking the β-galactosidase activity measured under inducing conditions and dividing by the basal level values.



	Fragment Length	Orientation	Basal Levels +	Basal Levels -	HU Induction Ratios +	HU Induction Ratios -
1	438 bp	-612 to -174	0.3	0.4	21	50
2	291 bp	-612 to -321	2.6	0.2	8	38
3	202 bp	-612 to -410	4	0.4	13	19
4	165 bp	-612 to -447	3	0.2	12	7
5	141 bp	-612 to -471	18	3	1	1
6	168 bp	-489 to -321	4	nd	14	nd
7	121 bp	-442 to -321	61	nd	1.3	nd
8	109 bp	-519 to -410	1.5	0.2	7	6
9	79 bp	-489 to -410	1	0.3	5	5

FIG. 3. 3′ deletion and subcloning analysis of the *RNR2* regulatory region. (A) Restriction map of the *RNR2* regulatory region; (B) various fragments taken from this region and subcloned into the UAS probe vector, pLG312 ΔSS (Fig. 1). Most fragments were cloned into the assay vector in both orientations as described in Materials and Methods. The positive orientation indicates that the fragment retains the same orientation relative to the start of transcription in the *CYC1* construct as in the native promoter; the negative orientation indicates that the fragment is placed in an inverted orientation relative to the start of transcription in the *CYC1* construct as in the native promoter. β-Galactosidase activities were measured for the yeast strain LN114 containing these constructs in the absence (basal levels) or presence (induced levels) of 100 mM HU. The induction ratios listed were calculated by dividing the induced levels of the basal levels of β-galactosidase activity. Fragments 1 through 5 all have the *Hin*dIII site as their 5′ borders and extend 3′ the indicated lengths. Fragment 6 uses the Δ24 deletion endpoint as its 5′ border and extends to the *Dde*I site. Fragment 7 uses the Δ30 deletion endpoint as its 5′ border and extends to the *Dde*I site. Fragment 8 uses a *Hpa*II site as its 5′ border and extends 109 bp to the *Rsa*I site. Fragment 9 uses the Δ24 deletion endpoint as its 5′ border and extends to the *Rsa*I site. nd, Not done.

sequence (URS1) resulted in an increase in the basal level of approximately 8-fold and a smaller (1.6-fold) increase in the induced level. A partial inactivation of URS1 may have occurred in the −470 deletion or there may exist two separate URS sequences, one between −489 and −470 and one between −470 and −460, a possibility that cannot be excluded in this study. Further evidence for URS1 is presented below (Fig. 3 and 8).

Evidence for the presence of a UAS was also seen in the 5′ deletion data [compare deletions −442(Δ30) and −403 (Δ22)]. Removal of this positive element reduced the basal level 10-fold and the induced level 7-fold. This positive element was repressed by the URS1 element described above. Further evidence supporting the existence of this UAS element is presented below (Fig. 4 and Table 2). The magnitude of the contribution of this UAS element to the normal basal level of *RNR2* cannot be determined from this analysis.

The modest and gradual reduction of both the basal and induced levels of *RNR2* expression resulting from the deletion of DNA located between −960 and −612 may suggest the presence of weak positively acting sequences in these regions. However, we were unable to detect UAS activity of this region when subcloned into pLG312 (data not shown). The relevance of the low basal level and low-level inducibility remaining after deletion of most of the promoter sequences (−403 to −110) is not understood and may reflect artifactual sequence context effects.

3′ deletion mutations define the DNA damage-responsive element (DRE) of *RNR2*. A series of 3′ deletions was created in the *RNR2* regulatory region by using convenient restriction sites and was subcloned between the *Bgl*II and *Xho*I sites on pLG312 ΔSS. Fragments were placed in both orientations on this vector, and yeast cells bearing these constructs were assayed for the ability to direct β-galactosidase expression in the presence and absence of treatment with HU (Fig. 3). Fragments from the *RNR2* promoter could confer DNA damage regulation on a heterologous promoter (*CYC1*). These fragments did show orientation effects, but the qualitative effects of a given deletion on a fragment were generally similar regardless of orientation. Orientation is described relative to the orientation in the native *RNR2* promoter.

The behavior of these subclones suggested the presence of a URS element located between or overlapping sequences −471 to −447 (Fig. 3; compare lines 4 and 5 and lines 6 and 7). This element, URS1, was able to repress UAS elements located both 5′ and 3′ to it. In addition to increasing the basal level of expression between 6- and 15-fold, the loss of URS1 appeared to completely abolish the ability of these fragments to confer HU inducibility on the *CYC1* promoter. These constructs were also tested for the ability to respond to DNA damage produced by methyl methanesulfonate, and results similar to those obtained with HU were observed for each clone (data not shown). These data suggest that URS1 may be intimately linked to the response to DNA damage and

confirm the results observed with the 5′ deletions on the native promoter concerning the existence and position of URS1.

As mentioned above, these subclones also indicated the existence of at least two separate UAS elements located on nonoverlapping DNA fragments (Fig. 3, compare lines 5 and 7). One element (UAS1) was found between −612 and −471 (line 5), and the second (UAS2) was found between −442 and −321 (line 7). Sequences defining UAS1 may actually contain two separate UAS elements, one suggested to lie between −612 and −519 that contributes to both the basal and induced levels (compare lines 3 and 8) and a second, weak UAS located between −489 and −447, defined by the D1 oligonucleotide (see Table 2 and Fig. 8). Since the UAS element suggested to lie between −612 and −519 has not yet been isolated on a DNA fragment containing only that DNA, it has not been formally proven to be a UAS element in and of itself (i.e., the deletion effects could be due to the increased proximity of vector sequences).

The UAS2 element defined by line 7 of Fig. 3 overlapped sequences implicated as a UAS by the 5′ deletions on the native promoter in Fig. 2 (−442 and −403). Both UAS1 and UAS2 were repressed by URS1 (Fig. 3; compare lines 5 and 4 and lines 7 and 6). UAS2 was not essential for the response to DNA damage because fragments lacking this sequence were inducible by HU (line 4), although it may have contributed to the basal and induced levels (compare lines 3 and 4).

A careful comparison of the effects of the various deletions shown in Fig. 3 reveals fluctuation in basal and induced levels suggestive of other positively and negatively acting sequences. However, these putative elements will not be discussed further because they need additional definition. One fairly large fluctuation (compare lines 1 and 2 in the positive orientation) was likely an artifact due to the deletion of the *RNR2* TATA sequence that is located 5′ to the *Xho*II restriction site. In that case, the basal β-galactosidase level in line 1 may not adequately reflect the level of transcription because there may exist transcripts that initiate 5′ to the normal *CYC1* starts and fail to present *lacZ* in the proper translational context (i.e., multiple upstream AUGs).

A 79-bp fragment (Fig. 3, line 9) was capable of conferring DNA damage regulation on a heterologous promoter (*CYC1*) and has been named the DRE. This DRE fragment derives its 5′ border from the Δ24 deletion and its 3′ border from the *Rsa*I site. It alone cannot account for all of the response to DNA damage (Fig. 3; compare lines 1 and 9), but it does contain sequences essential for this response and for this reason will be analyzed in greater detail.

Deletion analysis of the DRE of *RNR2*. To further define the *cis*-acting sequences important for the transduction of DNA damage information to the *RNR2* gene, a more detailed deletion analysis of the DRE was performed. To facilitate this analysis, three point mutations were introduced into the DRE by oligonucleotide synthesis: an A-to-C change at position −449, a C-to-G change at position −450, and an A-to-G change at position −451 (Fig. 4 and Table 1). These changes resulted in the creation of a *Not*I restriction site. The fragments used for the deletion analysis are shown in Fig. 4B, and their effects on β-galactosidase synthesis are listed in Table 2. It should be noted that the sequence in Fig. 4 differs from the published sequence (8, 11) by the insertion of a C at position −471 that was left out of the original sequence.

Introduction of the *Not*I site had no effect on the DRE function. The URS1 element defined above resided within or overlapping sequences between −489 and −468 because deletion of those 21 bp from the Δ24 border increased the basal level 16-fold (compare the D1,2 and Sty-Rsa clones). This deletion also greatly reduced the ability to respond to HU treatment. Deletion of an additional 16 bp (D2) had no significant effect on expression. However, a further deletion of 15 bp, producing the 26-bp fragment FII, completely abolished UAS function present on D2 and more precisely defined the location of UAS2 to within or overlapping −442 and −427. This location was also confirmed by the 3′ deletions (Fig. 4B; compare Δ24-62 and D1).

The basal level of D1 was low but retained DNA damage inducibility. Because D1 could promote transcription from a heterologous promoter, it contained a UAS element (UAS1). It also contained a URS element, and therefore both a positive and a negative effector function through this 42-bp D1 fragment. UAS2 as defined by D2 appeared to be repressible by URS1 and to contribute to both the basal and induced levels conferred by the DRE (compare D1, D2, and D1,2).

Binding of two distinct factors to sequences in the DRE of *RNR2*. In an attempt to define the biochemical components involved in sensing and transducing the DNA damage stress response, we began a search for proteins that interact with the *RNR2* DRE. A gel electrophoresis mobility shift assay was used to investigate binding of these factors to probe DNA. The analysis was performed with a 117-bp ^{32}P-labeled probe containing 79 bp of the DRE sequence from Δ24 to the *Rsa*I site (Fig. 4) (the additional 38 bp was derived from a polylinker used in manipulating this segment) and a yeast whole-cell extract. Two complexes were detected when extract was mixed with probe (Fig. 5). The fastest-migrating complex (band I) was due to a very abundant factor present in the extract because 100% of the probe was shifted in the presence of 1 μg of extract. The slower-migrating complex (band I+II) increased in abundance when more extract was added. Both complexes were competed for by excess unlabeled *RNR2* probe (only the fastest-migrating complex can be observed in the 2-μg lane in this exposure, but upon longer exposures the second complex could be detected and was efficiently competed for as well). When the 44-bp *Mnl*I fragment containing UAS2 was used as competitor, both complexes disappeared and a complex of intermediate mobility (band II) appeared, with a band intensity equal to that of band I+II (Fig. 5). We interpret this result to mean that the slowest-migrating complex contained two factors bound simultaneously. When the abundant factor I was fully competed for, the less abundant factor II could bind alone, resulting in a complex with a mobility intermediate between that of factor I bound alone and that of factors I and II bound together (band I+II). Neither complex was competed for by pUC19 DNA, demonstrating the specificity of the binding.

Mapping of the binding site of factor I. To identify the region of *RNR2* DNA to which factor I binds, we took advantage of the extensive deletion and subcloning analysis previously undertaken in the process of defining the DRE. Fragments derived from these molecules were used as competitors in gel electrophoresis mobility shift assays (Fig. 6). Using fragments derived from the deletions of Fig. 2, we observed that fragments extending from Δ30 to the *Dde*I site were efficient competitors for factor I binding (lane 9) but that the Δ22-*Dde*I fragment (lane 10) failed to demonstrate detectable competition. The 44-bp *Mnl*I fragment also competed, which limited the binding site for factor I to the DNA between the Δ30 deletion at −442 and the *Mnl*I site at −422. Although not shown in Fig. 6, fragment Δ24-62 (Fig. 4) also acted as an efficient competitor, placing the 3′ border at

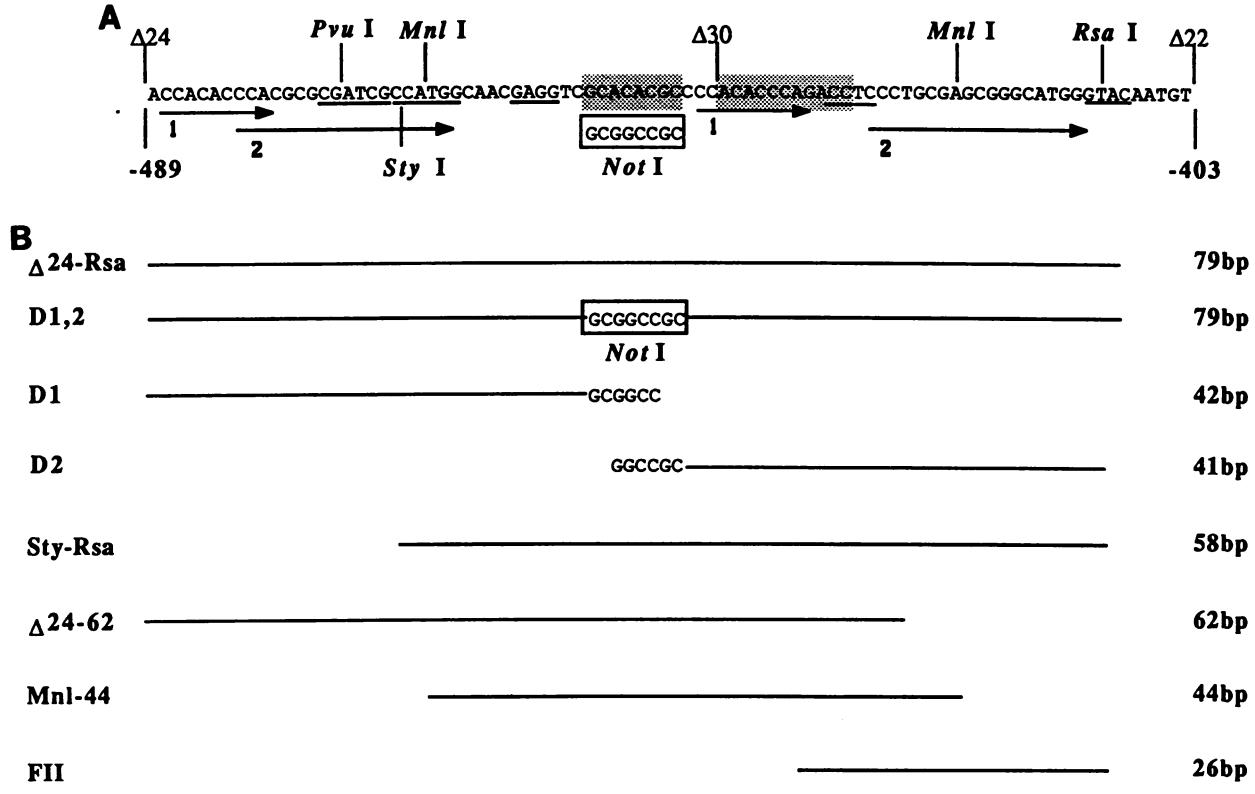


FIG. 4. Deletion analysis of the *RNR2* DRE. (A) Sequence of the region of the *RNR2* regulatory region between the deletion endpoints Δ24 and Δ22. Recognition sites for several restriction endonucleases are underlined, and the enzyme recognizing that sequence is listed above or below its site. The first shaded sequence indicates the location of three point mutations introduced into this sequence to create a *Not*I restriction enzyme recognition sequence as described in the text. The second shaded region indicates a potential recognition site for the DNA-binding protein GRF1. Numbered arrows underline sequences that are repeated in the DRE and serve as potential binding sites for *trans*-acting factors. (The sequence defined by the second arrow has several palindromic regions within it.) (B) Deletion fragments used. The names of the fragments are listed on the left, and their sizes are given on the right. The lines represent the DNA remaining in each of the deletions.

−427. This left a 15-bp region, −442 to −427, as the binding site for factor I. This site also precisely corresponds to the minimal region necessary for UAS2 function, suggesting that factor I is a positively acting transcription factor that interacts with UAS2 in vivo to promote transcription. It should be noted that the complex indicated as band I+II actually resolved into a doublet of bands in this experiment. Evidence that several other proteins interact with this fragment is given below (see Fig. 9).

TABLE 2. Functional analysis of DRE deletions[a]

Clone	Basal level for given orientation		HU induction ratio for given orientation	
	+	−	+	−
Δ24-Rsa	1.0	0.3	5	5
D1,2	1.2	ND[b]	4.5	ND
D1	0.3	ND	3.5	ND
D2	11.2	ND	0.9	ND
Sty-Rsa	16	8.6	1.2	1.3
Δ24-62	1.3	ND	10	ND
Mnl-44	4.7	1.4	1	1.3
FII	0	ND	0	ND

[a] DNA fragments described in Fig. 4 were cloned into pLG312 ΔSS, and their ability to direct β-galactosidase synthesis was measured in the absence or presence of 100 mM HU, as described in Materials and Methods.
[b] ND, Not done.

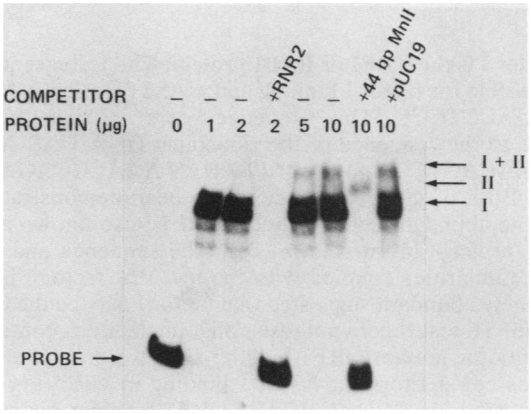


FIG. 5. Gel electrophoresis mobility shift assays performed with protein from whole-cell extracts to detect proteins that bind to the *RNR2* regulatory region. The probe was a ^{32}P-labeled *Bgl*II-*Xho*I fragment from pNN410 that contains the 79-bp DRE of *RNR2* and 22 bp of unrelated vector sequences. Binding reactions with competitor contained either 30 ng of the 312-bp *Hin*dIII-*Dde*I *RNR2* fragment, 15 ng of the 44-bp *Mnl*I fragment, or 30 ng of *Eco*RI-cleaved pUC19 DNA, as indicated. Reactions were performed as described in Mate rials and Methods and were analyzed by electrophoresis in a 4% polyacrylamide gel, which was then dried and autoradiographed. Mobilities of the various protein-DNA complexes are shown on the left.

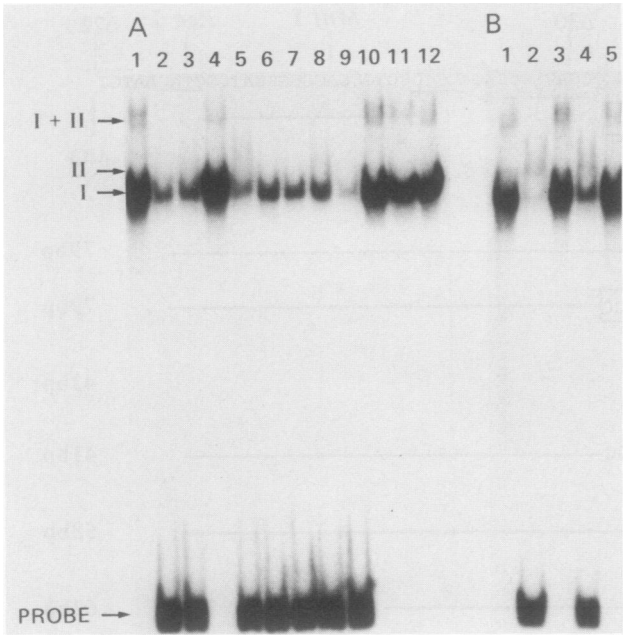


FIG. 6. Mapping of the binding site of factor I and identification of factor I as the GRF1 protein. (A) Gel electrophoresis mobility shift assays performed with 10 μg of protein from whole-cell extracts in the absence or presence of approximately 25 ng of specific competitor DNA. The probe was a ^{32}P-labeled *Bgl*II-*Xho*I fragment from pNN410 that contains the 79-bp DRE of *RNR2* and 22 bp of unrelated vector sequences. The presence of a band at the position labeled PROBE indicates successful competition for factor I binding. Lanes: 1, no competitor; 2, *Hin*dIII-*Xho*II fragment; 3, *Pvu*I-*Xho*II fragment; 4, *Hin*dIII-*Pvu*I fragment; 5, 44-bp *Mnl*I fragment; 6, *Pvu*I-*Rsa*I fragment; 7, Δ24-*Rsa*I fragment; 8, Δ24-*Dde*I fragment; 9, Δ30-*Dde*I fragment; 10, Δ22-*Dde*I fragment; 11, Δ360-*Dde*I fragment; 12, *Eco*RI-cleaved pUC19. (B) Competition assays using oligonucleotides with intact or mutant GRF1-binding sites. Lanes: 1, no competitor; 2, oligonucleotide ENO1; 3, oligonucleotide ENO1mtA; 4, oligonucleotide EII; 5, oligonucleotide EIImtA.

Factor I is the GRF1 or RAP1 protein. The 15-bp sequence responsible for factor I binding and UAS2 function is ACAC CCAGACCTCCC. This sequence bears a striking resemblance to the sequence of the ribosomal DNA UAS ACAC CCAAACACTCG and the *MAT*α UAS AAACCCAGACAT CAT. Both of these sequences have been demonstrated to bind the abundant cellular factor GRF1 (also known as the RAP1 protein) in vitro (5, 6, 21). The sequence and functional similarities coupled with the fact that factor I is also extremely abundant suggested that factor I may be the GRF1 protein. To test this hypothesis, oligonucleotides containing authentic or mutant GRF1-binding sites were obtained and used as competitors for factor I binding in vitro (Fig. 6B). Oligonucleotide ENO1 is a 38-bp GRF1-binding site derived from *ENO1*, the gene encoding enolase, and the 31-bp oligonucleotide EII was derived from the GRF1-binding site in the silencer at *HMRa* (6). The sequences of these oligonucleotides and their mutant derivatives are illustrated in Table 1. Oligonucleotide ENO1 was a better competitor than oligonucleotide EII, but both acted as efficient competitors for factor I binding. The ENO1 site was also an eightfold better competitor than EII for GRF1 binding (6). However, ENO1mtA and EIImtA, mutant derivatives that also failed to bind GRF1, had no detectable competitor activity. These data suggest that factor I is GRF1.

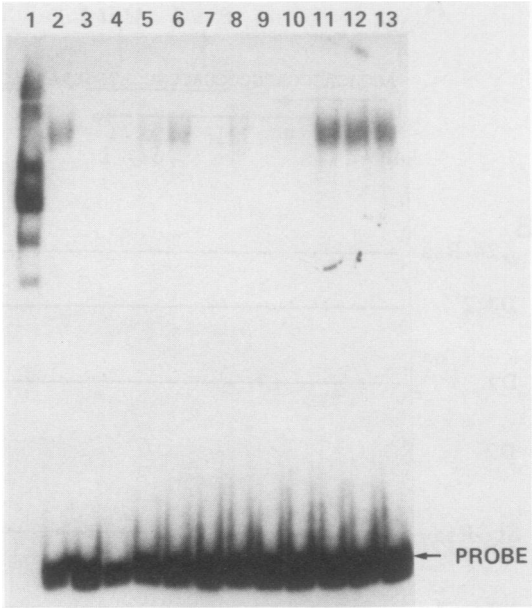


FIG. 7. Mapping of the binding site of factor II. Gel electrophoresis mobility shift assays were performed with 10 μg of protein from nuclear extracts in the absence or presence of approximately 25 ng of competitor DNA fragments and 50 ng of oligonucleotide ENO1. The probe was a ^{32}P-labeled *Bgl*II-*Xho*I fragment from pNN410 that contains the 79-bp DRE of *RNR2* and 22 bp of unrelated vector sequences. Lanes: 1, no competitor; 2, 50 ng of ENO1 competitor; 3 to 13, 50 ng of ENO1 as competitor in addition to the following: *Hin*dIII-*Xho*II fragment (lane 3), *Pvu*I-*Xho*II fragment (lane 4), *Hin*dIII-*Pvu*I fragment (lane 5), 44-bp *Mnl*I fragment (lane 6), *Pvu*I-*Rsa*I fragment (lane 7), Δ24-*Rsa*I fragment (lane 8), Δ24-*Dde*I fragment (lane 9), Δ30-*Dde*I fragment (lane 10), Δ22-*Dde*I fragment (lane 11), Δ360-*Dde*I fragment (lane 12), and *Eco*RI-cleaved pUC19 (lane 13).

Mapping of the binding site of factor II. The inclusion of oligonucleotide ENO1 in the binding reactions efficiently competed for factor I binding and allowed the analysis of factor II binding. The same deletion fragments used in the mapping of the factor I-binding site were used as competitors for factor II binding in the presence of 50 ng of oligonucleotide ENO1 (Fig. 7). Nuclear extracts were used in this experiment because they contained higher levels of factor II, although they showed extensive proteolysis of GRF1 (Fig. 7, lane 1). As observed for factor I binding, fragments extending from Δ30 to the *Dde*I site were efficient competitors for factor II binding (lane 10). However, the fragments extending from Δ22 to the *Dde*I site failed to demonstrate detectable competition (lane 11). This result places sequences important for factor II binding between Δ30(−442) and Δ22(−403). The 44-bp *Mnl*I fragment showed weak but detectable levels of competition, suggesting that the factor II-binding site may lie on the 5′ half of the region from −442 to −403. Furthermore, the *Hin*dIII-*Pvu*I fragment also showed some weak competition (lane 5), suggesting that there may be more than one binding site for factor II.

Both a negative and positive element exist within the 42-bp D1 fragment. The deletion analysis of the DRE suggested that the 42-bp D1 fragment could act to confer damage inducibility upon a heterologous promoter. It also suggested that a negative regulatory element existed within this fragment. To test this hypothesis, the D1 fragment was placed adjacent to a fragment containing the α1 UAS (5, 6). The

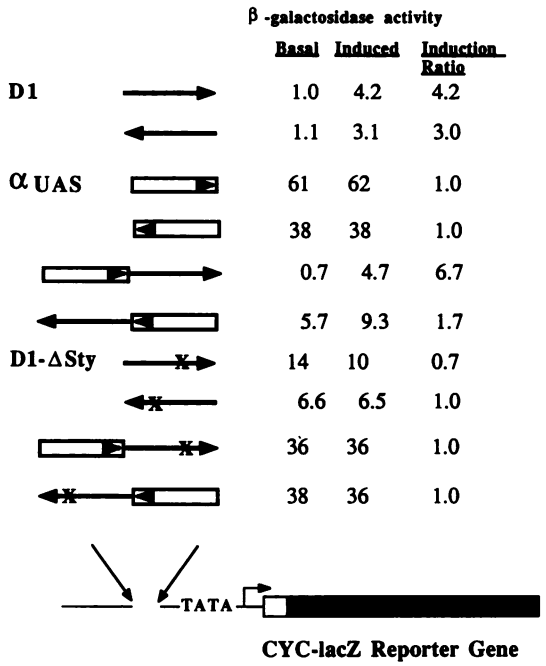


FIG. 8. Demonstration that the D1 fragment contains both an activator and a repressor of transcription. The D1 fragment or a mutant form, D1-Δ*Sty*I, was placed either alone or in combination with an α UAS fragment into pLG312 ΔSS *Bgl*II. β-Galactosidase activities were determined from yeast strains containing these constructs in the absence (basal) or presence (induced) of 100 mM HU. Arrows represents the D1 fragment; the direction of each arrow indicates the orientation of the fragment. The box with an arrowhead represents the α UAS. The arrow with an X represents the mutant fragment D1-Δ*Sty*I. This 4-bp insertion mutation was created by cleaving the D1 fragment with *Sty*I and filling in the site with T4 polymerase to create an *Nsi*I site. At the bottom is a schematic representation of the position of insertion of these fragments in front of the *CYC1-lacZ* reporter gene.

ability to direct transcription of a heterologous promoter was measured for these elements alone or in combination (Fig. 8). The D1 element gave a slightly higher basal level in this particular construct than in the previous construct shown in Table 2, presumably because of spacing and context differences. The D1 fragment could act as a repressor of the α1 UAS when placed on either side of the element, although it was a more efficient repressor when located between the UAS and TATA elements. The DNA damage induction mediated by D1 was not greatly affected by the presence of the α1 UAS with respect to the increase in the absolute amount of expression. The presence of HU did not appear to release repression of the α1 UAS by D1.

A 4-bp insertion mutation was introduced into D1 by cleaving the fragment with *Sty*I and filling in with T4 polymerase. This mutation appeared to eliminate the ability of D1 to repress the α1 UAS and increased its own basal level of expression. This mutation had either destroyed the ability of a repressor to bind this element, altered the required spacing within this element, or created a binding site for a new positively acting factor. Further analysis is necessary to distinguish among these possibilities.

Existence of two binding sites for factor II and a single binding site for two new proteins, factor III and IV, in the *RNR2* promoter. To identify additional factors responsible for the damage inducibility of *RNR2*, factors that bound the D1 fragment were examined. Three species of retarded mobility were observed when labeled D1 was incubated with protein extracts (Fig. 9A). The D1 fragment itself competed efficiently for only two of the three species (Fig. 9A, lane 3) at the competitor concentrations used in this experiment. In lane 2, the competitor DNA had single-stranded ends produced by the restriction enzyme, whereas the probe was made flush by filling in those ends with Klenow fragment and deoxyribonucleoside triphosphates. However, filling in the ends of the competitor did not increase its ability to compete for the lower band (complex III; data not shown). The binding site that was responsible for protein-DNA complex II was not an artifact created by the polylinker sequences on the probe because fragments from the native promoter (Δ24-*Rsa*I [lane 5] and Δ30-*Dde*I [lane 6] but not Δ22-*Dde*I [lane 7]) acted as efficient competitors for the factor(s) that interacted with that site. Interestingly, the Δ30-*Dde*I fragment does not overlap with the sequences in the probe, indicating that two separate binding sites for this factor exist within the *RNR2* promoter. The 4-bp insertion mutation in D1 that interfered with its ability to repress the α1 UAS behaved identically to the native D1 fragment when used as a competitor (lane 3) or as a probe for gel shift experiments (data not shown).

The protein-DNA complex of intermediate mobility (complex II) was competed for by the Δ24-*Rsa*I fragment (Fig. 9A, lane 5) and by the smallest D1 fragment itself (lane 8) but not by the Δ30-*Dde*I fragment (lane 6). This result suggested that it was a new factor which bound to a single site in the interior of the D1 element. This factor should have been identified as a factor that bound to the DRE but could not be competed for by the Δ30-*Dde*I fragment. Such a factor was not observed in Fig. 7. One possible explanation is that the abundance of this factor is fairly low and may have been observed under other conditions. A second possible explanation is that the specific competitor oligonucleotide ENO1 used in that experiment to compete for factor I binding also competed for binding of this factor. There are sequence similarities between sequences in oligonucleotide D1 and the GRF1-binding site on oligonucleotide ENO1. This hypothesis is now being tested. A third possibility is that this factor was lost or destroyed during preparation of the nuclear extract.

The protein-DNA complex of slowest mobility (complex I) was competed for efficiently by excess D1 fragment (Fig. 9A, lane 3) but poorly by the native Δ24-*Rsa*I fragment (lane 5). However, the minimal 42-bp D1 sequence defined by oligonucleotide D1 (Fig. 4) did not compete for this complex (lane 8), suggesting that the protein-binding site for that complex resides near one end of the D1 fragment.

Since the D1 sequence differs from the native sequence by three bases used to create the *Not*I site, it was possible that these changes affected the binding of some of the proteins detected in Fig. 9A. To test this possibility, we used as a probe a second oligonucleotide (Dxb; Table 1) that contained a native *RNR2* sequence covering the region altered during creation of the *Not*I site. The three protein-DNA complexes detected with this sequence used as a probe (Fig. 9B) had mobilities nearly identical to those observed with D1 used as a probe when run side by side (data not shown) and similar affinities with one striking exception: the abundance of protein-DNA complex I was greatly diminished relative to levels of complexes II and III. We interpret this result to mean that the protein that bound D1 and Dxb to form complex I interacted with sequences at the 3′ end of D1 near the *Not*I site and that the changes made to create the *Not*I

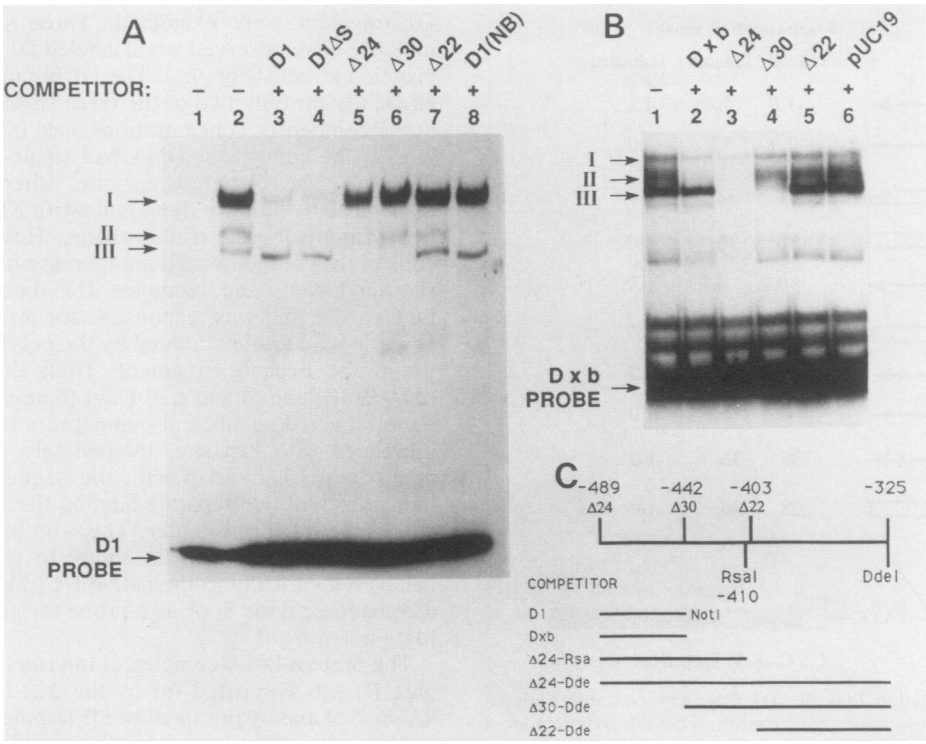


FIG. 9. (A) Gel electrophoresis mobility shift assays performed with protein from whole-cell extracts to detect proteins that bind to the D1 fragment. The probe was a ^{32}P-labeled *Eco*RI-*Xho*I fragment from pNN411 that contains the 42-bp D1 fragment and 22 bp of unrelated polylinker sequences. Binding reactions with competitor contained approximately 25 ng of competitor DNA. Lanes 3 through 8 contain competitor DNA as indicated. Lanes: 1, no extract added; 2, no competitor; 3, D1, an *Eco*RI-*Xho*I fragment of pNN411; 4, D1-Δ*Sty*I, an *Eco*RI-*Xho*I fragment of pNN412; 5, *Bgl*II-*Xho*I fragment of pNN410 (Δ24-*Rsa*I); 6, Δ30-*Dde*I fragment (Fig. 2); 7, Δ22-*Dde*I fragment (Fig. 2); 8, *Bgl*II-*Not*I D1 fragment of pNN411 (this is equivalent to the sequence of the D1 oligonucleotide presented in Table 1). Reactions were performed as described in Materials and Methods and were analyzed by electrophoresis in a 4% polyacrylamide gel, which was then dried and autoradiographed. Mobilities of the various protein-DNA complexes are indicated on the left. (B) Gel electrophoresis mobility shift assays performed with protein from whole-cell extracts to detect proteins that bind to the Dxb fragment. The probe was a ^{32}P-labeled Dxb fragment containing the 47-bp Dxb fragment and 20 bp of unrelated polylinker sequences as described in Materials and Methods. Binding reactions with competitor contained approximately 25 ng of competitor DNA. Lanes: 1, no competitor; 2, the same Dxb fragment as was used as a probe; 3, Δ24-*Dde*I fragment; 4, Δ30-*Dde*I fragment; 5, Δ22-*Dde*I fragment; 6, 166-bp *Dde*I fragment from pUC19. (C) Fragments of DNA used as competitors relative to a restriction map of the region.

site increased the affinity for this factor. Alternatively, two different factors could have interacted with D1, and the more abundant factor failed to bind the native sequence (Dxb). In addition to these three bands, a fourth band of faster mobility was seen to be competed for by Dbx and Δ24-*Dde*I. However, the pattern of competition for this complex was the same as that for complex II, and the complex may have been a degradation product of the protein that bound to form complex II. Since it cannot be formally distinguished from complex II, this band will not be discussed further.

The competition with native promoter fragments gave a similar pattern of results for Dxb as for the D1 probe. The Dxb fragment gave weak but detectable competition for complex III (Fig. 9B; compare band III in lane 2 with band III in lanes 1, 5, and 6). The degree of reduction was approximately 25 to 50%. In addition to competing for complex III, the Δ30-*Dde*I fragment appeared to have slightly reduced complex II in this experiment (compare lane 4 with lanes 1, 5, and 6). This result may have been due to the complete loss of the dark band III; this band partially overlapped band II, a fact that may have contributed to its darkness. We cannot rule out the possibility that there was an additional binding site between Δ30 and Δ22 for the factor that bound to form complex II. If there was an additional site, it was much weaker than the site present on Dbx, since both Dbx and Δ24 were much better competitors on a molar basis than was Δ30. Regardless, all three complexes were specifically and differentially competed for by native *RNR2* promoter DNA (Fig. 9A, lanes 5 to 8; Fig 9B, lanes 3 to 5) and not by nonpromoter DNA (Fig. 9B, lane 6) and thus define three additional distinct factors in addition to GRF1 that bind to the DRE of *RNR2*. These factors have been tentatively named RRF1, -2, and -3, for ribonucleotide reductase regulatory factor. RRF1 binding is responsible for complex I, RRF2 binding is responsible for complex II, and RRF3 binding is responsible for complex III.

DISCUSSION

Sequences important for the regulation of *RNR2*. We have analyzed the region of DNA upstream of the *RNR2* gene in an attempt to identify regions important for the regulation of *RNR2*. Regulatory elements controlling gene expression in yeast cells generally lie within 400 bp of the TATA region of a given promoter (4–6). The upstream region of the *RNR2* gene is characterized by a G+C-rich (60%) region extending over a 150-bp region (−489 to −339 [−270 to −120 relative to the TATA]), much like the regulatory regions of the *GAL1* and *GAL10* promoters (14).

Our results indicate that sequences within this G+C-rich region are important for *RNR2* expression. 5′ deletion anal-

ysis combined with subcloning analysis revealed the existence of both activating and repressing sequences within this region and potentially upstream of it. They furthermore showed that sequences from this region could confer DNA damage inducibility upon the heterologous *CYC1* promoter. A model summarizing the positions of sequences determined to contain transcriptional information within the *RNR2* regulatory region is shown in Fig. 10A. The existence of UAS1 is supported primarily by two pieces of evidence. First, the 141-bp *Hin*dIII-*Pvu*I fragment can activate a *CYC1* promoter that is deleted for its own UAS elements (Fig. 3, line 5). Second, the D1 fragment can also activate the disabled *CYC1* promoter (Table 2 and Fig. 8). This second fact localizes UAS1 to sequences −489 to −447 and potentially between −489 and −471. This is a small region of overlap, and it is entirely possible that both fragments direct transcription by using different UAS elements. We feel that it is highly likely that another UAS (UAS3) exists between −612 and −519, a view supported by the difference in transcriptional potential between fragments from lines 3 and 8 in Fig. 3. However, isolation of this fragment alone and demonstration that it can direct transcription of a heterologous promoter must be accomplished before UAS function can be proven.

The existence of a second UAS (UAS2) is also supported primarily by two lines of evidence. First, the deletion of 39 bp between −442 and −402 (Δ30 and Δ22; Fig. 2) reduces both basal and induced levels of *RNR2* expression. Second, the D2 fragment containing sequences from −451 to −410 is capable of directing transcription of a heterologous promoter (Table 2).

The existence of repressing sequence URS1 is supported by several lines of evidence. First, deletions to −470 and −442 (Δ30) (Fig. 2) increase the basal level of transcription of *RNR2*, suggesting the loss of a negative regulatory element that is located between or overlapping −489 and −470 to −442. That this effect is not due to the proximity of vector sequences was demonstrated by similar results obtained in the subcloning deletion analysis, in which a totally different assay was used with different adjacent vector sequences. In those experiments, removal of DNA between −471 and −447 (Fig. 3, lines 4 and 5) or −489 and −442 (lanes 6 and 7) caused an increase in the basal level of expression of the *CYC1* promoter. The final line of evidence is that the D1 fragment is capable of repressing the α1 UAS (Fig. 8).

Factors that bind the *RNR2* regulatory region. We have characterized four yeast DNA-binding factors that interact with the 79-bp DRE in an effort to identify the proteins mediating the damage response. These factors are thought to be specific DNA-binding proteins because the formation of their particular complexes is specifically competed for by native *RNR2* promoter sequences and not by other DNAs such as pUC19 fragments or various other fragments of the *RNR2* promoter that do not contain binding sites. The relative positioning of these binding factors is illustrated in Fig. 10B. One factor has been identified as the GRF1 protein (5, 6), the product of the *RAP1* gene (21), and binds the element defined as UAS2 by deletion analysis. Although GRF1 can act as both a negative and a positive regulator of gene expression, depending on its environment, it appears to act as a positive element in the *RNR2* promoter. As noted above, we have tentatively named the other three factors RRF1, RRF2, and RRF3. RRF3 has two binding sites in the DRE, as defined by competition for binding by nonoverlapping contiguous segments of DNA. At least two distinct regions within the left half of the DRE show sequence

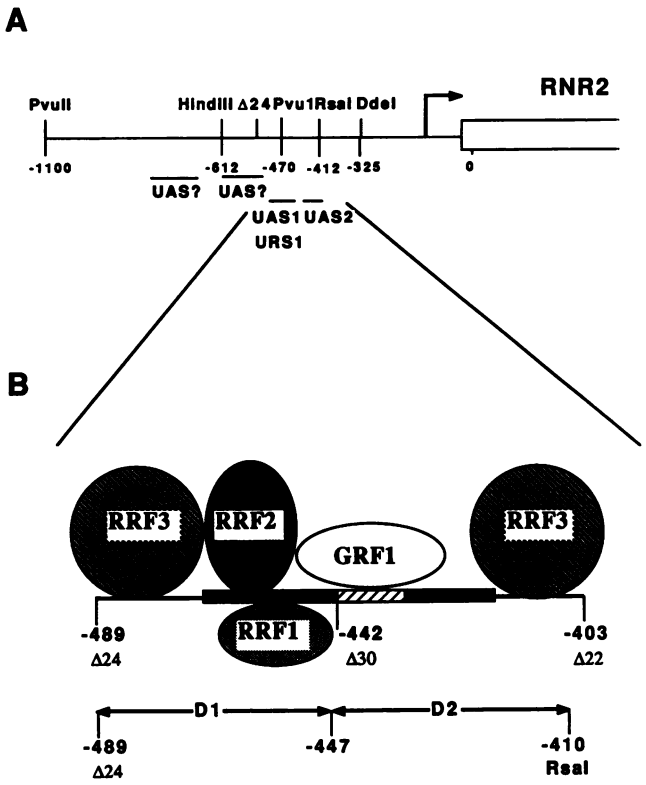


FIG. 10. Locations of functional elements within the *RNR2* promoter region. (A) Restriction map of the *RNR2* gene. Below the map are indicated the positions of UASs or URSs identified in this study. UAS?, Sequences that appear to influence levels of *RNR2* expression when deleted but which alone have not demonstrated the ability to act to promote expression of the *CYC1-lacZ* fusion or that have not been separated from other defined UAS elements. (B) Expanded section of the *RNR2* promoter region corresponding to the DRE showing the approximate positions of DNA-binding proteins identified in this study. Factor I is represented by the oval labeled RRF1; factor II is represented by the oval labeled RRF2; factor III is represented by the sphere labeled RRF3. The precise locations of binding are known only for GRF1 and RRF1. RRF2 is known to bind only to the left half of this fragment. RRF3 has binding sites in both halves of the DRE.

similarities with the right half of the DRE and serve as potential binding sites for RRF3 (Fig. 4). One such repeat overlaps the GRF1-binding site and may suggest a means of repression. At this level of analysis, it is not possible to precisely define the position of the binding sites for these factors. A binding site for RRF2 and RRF3 exists on the left half of the DRE (5′ to the Δ30 deletion), a binding site for RRF1 resides near the middle (the 3′ end of the D1 fragment), and a binding site for GRF1 and RRF3 exists on the right half of the element (3′ to the Δ30 deletion). The fact that RRF3 binds D1 but D1 is a poor competitor for that binding relative to native promoter fragments such as Δ24-*Rsa*I or Δ30-*Dde*I (Fig. 10) suggests that the binding site on the right half of the DRE has a much higher affinity for RRF3 than does the site on the left half. Alternatively, other cellular factors may bind to Δ24-*Rsa*I or Δ30-*Dde*I and facilitate or stabilize binding of RRF3.

The GRF1 protein does not play an essential role in the damage response, since induction can occur in deletions lacking it. If GRF1 is the only positively acting factor binding between −451 and −410 (the D2 fragment), then it is likely to

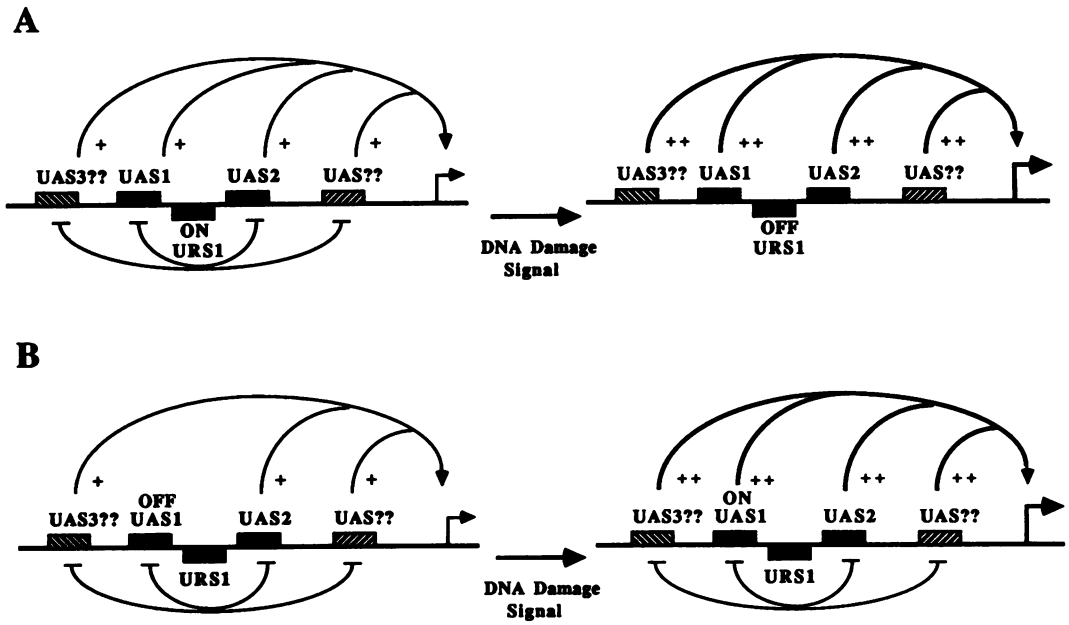


FIG. 11. Models of DRE function. (A) Repressor-mediated model for *RNR2* regulation by DNA damage. Symbols and abbreviations: ——, *RNR2* promoter DNA; labeled boxes, various sequences defined in this study to be involved in *RNR2* transcription; UAS3??, the position of a probable UAS discussed in this work; UAS??, other potential positive regulatory elements that may exist in this promoter but have not been formally defined; +, contributions to *RNR2* transcription; arcs joining together above the UASs, synergistic contributions to *RNR2* expression; arcs emanating from the URS and ending at the UAS elements, repression of those elements. (B) Activator-mediated model for *RNR2* regulation by DNA damage.

contribute to both the basal and induced levels of transcription (Table 2; compare D1,2 and D1). The roles of RRF1, -2, and -3 remain to be determined. The fact that the Δ30 deletion retains one RRF3-binding site and also retains some DNA damage inducibility is circumstantial evidence that it plays a role in this response. The fact that the induction is lost in the next deletion, which removes both the GRF1 and RRF3 sites, is supporting evidence for this hypothesis. The fact that the Δ*Sty*I insertion mutation appears to interfere with the action of a repressor function but does not appear to alter the binding of RRF1, -2, or -3 suggests that a fourth, as yet undetected factor may exist. Alternatively, the spacing among these elements may be critical for proper regulation, or perhaps the repressing sequence does not require a protein factor to mediate its repressing influence. Presumably, at least one of these three factors is responsible for the UAS1 activity of the 42-bp D1 fragment.

Models for the induction of *RNR2* expression by DNA damage. What sequences are necessary for damage inducibility of *RNR2*? We have shown that a small fragment of 42 bp (D1) can confer DNA damage inducibility upon a heterologous promoter. Although this region of DNA appears to be necessary and sufficient for some response to the stress signals produced by blocking replication (HU) or by DNA damage (methyl methanesulfonate; data not shown), it does not confer the same extent of induction observed to be effected either by the native promoter or by larger *RNR2* fragments placed in front of the *CYC1* promoter. This finding suggests that other promoter elements outside of this region are involved in the response; if not directly transducing the cellular response signal, they can contribute to the magnitude of the response by acting synergistically with those elements that do.

These results are consistent with two models for DRE function (Fig. 11). In the first model (Fig. 11A), a negative regulatory element, URS1, positioned on D1, represses the activity of several positive elements in the *RNR2* promoter, including UAS1 and -2 and other potential UAS elements. In this model, the protein interacting with URS1 is the recipient of DNA damage information, the presence of which diminishes its capacity to repress UAS1 and -2, resulting in induction of transcription. A second model (Fig. 11B) consistent with these data is that the UAS1 element on D1 interacts with a protein that becomes a transcriptional activator only in the presence of DNA damage. In this model, URS1 must repress UAS2 and potentially other UASs to provide a low basal level of expression so that the transcription induced from UAS1 can be detected. The activated UAS1-binding protein can act synergistically with other UAS-binding proteins bound to the *RNR2* regulatory region to magnify the effect of its activation. In this model, DNA damage induction still occurs in mutants lacking URS1 but cannot be detected because of the high basal level caused by the derepression of UAS function. URS1 has no direct role in DNA damage response but may play a role in some other regulatory circuit that we have yet to uncover, such as meiotic regulation or spore germination.

It seems likely that the DNA damage inducibility is mediated via the URS1 element as suggested in the first model for two reasons: (i) inclusion of this fragment with 3′ or 5′ nonoverlapping flanking sequences confers regulation on those fragments while repressing their basal levels of expression and (ii) the levels of expression of these fragments under inducing conditions is consistent with their recovering part of the transcriptional potential shown by the fragment in the absence of the URS1 element, as opposed to the small increase in transcription conferred by the 42-bp fragment alone. However, it is still possible that *RNR2*

regulation is mediated via a weakly acting positive regulatory element that is activated in the presence of DNA damage. When activated, this element could act synergistically with flanking 3′ and 5′ elements to increase transcription levels far beyond the capacity of these elements alone, as detailed in the second model. These are the two simplest models; more complex models can be designed to explain these results, especially in the absence of an understanding of how eucaryotic activators and repressors work. The inability of the 42-bp fragment to allow the full transcription potential of the α1 UAS element under the inducing condition suggests that orientation and spacing of regulatory elements may be important for proper regulation. Alternatively, a complementarity may exist between regulatory elements such that certain classes of transcription factors can be acted upon only by certain classes of regulatory factors. Furthermore, we have already seen that one protein factor (RRF3) binds multiple sites in the *RNR2* promoter, suggesting that some information may be redundant in the promoter. It is certainly possible that there are several independent elements that can individually respond to subsets of cellular distress signals. In addition, a combination of these models may explain the regulation, because their features are not mutually exclusive.

In an analysis of this nature, any given result is prone to potential artifacts due to contextual effects created by the proximity of vector sequences in vivo or DNA ends in vitro. We have tried to base our conclusions primarily on results that have been confirmed by two independent types of experiments, particularly in the deletion analysis. The analysis presented above has demonstrated that the regulatory region of the *RNR2* gene is extremely complex, containing binding sites for multiple *trans*-acting factors. These factors are likely to play an intimate role in transmitting the signal generated by DNA damage to the transcriptional machinery controlling *RNR2* expression. The next level of analysis of the *RNR2* promoter requires identification of the precise location of binding sites for these factors. This analysis will facilitate the use of site-directed mutagenesis to remove these binding sites in order to discover the in vivo role of each of these factors in the damage response. This information will determine whether we have identified all of the important factors in the regulation of *RNR2* and which factors should be pursued for the purpose of isolating regulatory genes mediating this response to DNA damage.

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