MOLECULAR AND CELLULAR BIOLOGY, Oct. 1997, p. 6105–6113 Vol. 17, No. 10
0270-7306/97/$04.00+0

Identification of *RNR4*, Encoding a Second Essential Small Subunit of Ribonucleotide Reductase in *Saccharomyces cerevisiae*

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Received 3 February 1997/Returned for modification 26 March 1997/Accepted 24 June 1997

Ribonucleotide reductase (RNR), which catalyzes the rate-limiting step for deoxyribonucleotide production required for DNA synthesis, is an $\alpha_2\beta_2$ tetramer consisting of two large and two small subunits. *RNR2* encodes a small subunit and is essential for mitotic viability in *Saccharomyces cerevisiae*. We have cloned a second essential gene encoding a homologous small subunit, *RNR4*. *RNR4* and *RNR2* appear to have nonoverlapping functions and cannot substitute for each other even when overproduced. The lethality of *RNR4* deletion mutations can be suppressed by overexpression of *RNR1* and *RNR3*, two genes encoding the large subunit of the RNR enzyme, indicating genetic interactions among the *RNR* genes. *RNR2* and *RNR4* may be present in the same reductase complex in vivo, since they coimmunoprecipitate from cell extracts. Like the other *RNR* genes, *RNR4* is inducible by DNA-damaging agents through the same signal transduction pathway involving *MEC1*, *RAD53*, and *DUN1* kinase genes. Analysis of DNA damage inducibility of *RNR2* and *RNR4* revealed partial inducibility in *dun1* mutants, indicating a *DUN1*-independent branch of the transcriptional response to DNA damage.

Ribonucleotide reductases (RNRs) catalyze the reduction of all four ribonucleoside diphosphates into deoxyribonucleoside diphosphates and play a central role in controlling the levels of cellular deoxynucleoside triphosphates (dNTPs), which are essential for high-fidelity DNA replication and DNA repair processes. Three classes of RNR enzymes have been characterized; they have unrelated protein structures, but all contain an organic free radical that is crucial for the catalytic activity, and all are allosterically regulated (for reviews, see references 23 and 31).

Class I enzymes are the most commonly found and best studied. They exist in all eukaryotes and in most prokaryotes, with the aerobic *Escherichia coli* RNR as the prototype. The functional class I enzyme complex is a heterotetramer ($\alpha_2\beta_2$) between two nonidentical homodimers (20), the large subunit (α or R1) and the small subunit (β or R2), each of which is inactive by itself. Each monomer of the R1 dimer (two 90-kDa monomers) contains one substrate-binding site with redox-active thiols and two separate allosterically regulated binding sites for dNTPs. One site binds to ATP and dATP and controls the overall enzyme activity to ensure the proper balance between the level of NTPs for RNA synthesis and that of dNTPs for DNA synthesis; the other site controls the substrate specificity to maintain balanced dNTP pools that are necessary for high-fidelity DNA replication. Each monomer of the R2 dimer (two 45-kDa monomers) contains a tyrosyl free radical that is essential for catalytic activity. This tyrosyl free radical is generated and maintained by an oxygen-linked binuclear ferric iron center buried inside the protein (22). The chemotherapeutic agent hydroxyurea (HU) scavenges this tyrosyl radical and thereby inactivates RNR activity (2). The flexible carboxyl-terminal tail of the R2 protein is essential for binding to the R1 protein and assumes a more rigid structure upon binding (18).

The localization and assembly of the RNR subunits appear to be differentially regulated. Immunocytochemical studies showed that the R1 and R2 subunits of the mammalian RNR enzyme are both localized in the cytoplasm (11) and in some cell types appear to be concentrated near the nuclear region (28). The R2 of the herpes simplex virus type 1 RNR enzyme is concentrated in discrete cytoplasmic foci close to the nucleus, whereas R1 exhibits a more diffuse cytoplasmic localization (4). The T4 phage RNRs are found in a multienzyme complex that is called dNTP synthetase complex, which is located near the sites of DNA replication (37).

Two common features are shared by the RNRs from all organisms examined. First, both the expression and the enzymatic activity of the RNR enzymes fluctuate during the cell cycle, with maximal activity in S phase (3, 7, 9, 10, 12, 13, 33). Second, the expression of RNR genes is inducible by DNA-damaging agents or replication blocks (6, 7, 14), although the mechanisms for such regulation may differ. Such conservation in regulation of RNR activity through evolution underscores the importance of dNTP level regulation to cellular function.

There are three known genes encoding RNR subunits in *Saccharomyces cerevisiae*, *RNR1*, *RNR2*, and *RNR3*. The large subunit is encoded by two highly homologous genes, *RNR1* and *RNR3* (8). *RNR1* is essential for mitotic viability, whereas *RNR3* is nonessential. *RNR1* transcription is tightly cell cycle regulated and moderately inducible by DNA damage. The *RNR3* transcript level is low under normal conditions but is highly inducible by DNA damage, increasing up to 100-fold (8). The small subunit is encoded by *RNR2*, which is both essential and DNA damage inducible (5). Transcriptional regulation of the RNR genes has been extensively studied; however, little is known about the stoichiometry of the RNR enzyme complex or the regulation of its assembly or activity in vivo.

Here we report the isolation and characterization of a second gene encoding an RNR small subunit from *S. cerevisiae*, *RNR4*. Our interest was drawn to *RNR4* initially because a missense mutation in it results in constitutively high transcrip-

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
Y203	*MAT***a** *ade2-1 his3 leu2-3,113 lys2 trp1 ura3-Δ100 rnr3::RNR3-URA3-TRP1*	38
Y205	*MAT*α *ade2-1 his3 leu2-3,113 lys2 trp1 ura3-Δ100 rnr3::RNR3-URA3-LEU2*	39
Y254	As Y205, *dun1-3*	39
Y300	*MAT***a** *can1-100 ade2-1 his3-11 leu2-3,112 trp1 ura3-1*	1
Y323	*MAT***a**/α *can1-100/can1-100 ade2-1/ade2-1 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1/trp1 ura3-1/ura3-1*	1
TWY397	*MAT***a** *ura3 his7 leu2 trp1*	36
TWY312	*MAT***a** *mec2-1* (*rad53-1*) *ura3 trp1*	36
TWY308	*MAT***a** *mec1-1 ura2 trp1*	36
Y566	As Y203, *rnr4-99*	This study
Y291	As Y203, *rnr4-99 dun1-3*	This study
Y567	As TWY397, *dun1-Δ100::HIS3*	This study
Y568	As Y300, *rnr4-99*	This study
Y569	As Y300, *rnr4-99 dun1-Δ100::HIS3* + pMH140 (*URA3*)	This study
Y570	As Y300, *rnr4-99 rad53-21* + pMH140 (*URA3*)	This study
Y571	As Y300, *rnr4-99 mec1-21* + pMH140 (*URA3*)	This study
Y572	As Y323, *RNR4*/*rnr4-Δ127::LEU2*	This study
Y573	As Y323, *RNR2*/*rnr2-312::TRP1*	This study
Y574	Y572 + pBAD70 (*TRP1*, *GAP-RNR1*)	This study
Y575	Y572 + pBAD79 (*TRP1*, *GAP-RNR3*)	This study
Y576	Y572 + pBAD52 (*TRP1*, *GAP*)	This study

tion of *RNR3* in the absence of any DNA damage (Crt phenotype [38]). We show that both *RNR2* and *RNR4* are essential for mitotic viability and may be present in the same RNR enzyme complex. We also show that the lethality of *RNR4* null mutations can be suppressed by overexpression of *RNR1* or *RNR3*, suggesting that the large subunit may be the rate-limiting factor in the assembly of functional RNR enzyme.

MATERIALS AND METHODS

Strains. The yeast strains used in this study are listed in Table 1. Y568 was obtained by outcrossing the *rnr4-99* allele six times into Y300.

Cloning of *RNR4*. A yeast genomic library in a *TRP1* centromeric (*CEN*) plasmid (a gift from F. Spencer) was transformed into Y216 and selected on SC-tryptophan–5-fluoroorotic acid (5-FOA) plates. Two plasmids with 3.4 kb of overlapping genomic sequence on chromosome VII, pMH77 and pMH78, were recovered in *E. coli*, reintroduced into Y216, and found to complement the Crt$^-$ phenotype. The Crt$^-$-complementing activity was further localized to a 2.7-kb *Nhe*I-*Avr*II fragment present on a *CEN* plasmid, pMH131 (*HIS3*). The sequence of the 3.4-kb genomic DNA was obtained from the *S. cerevisiae* genome database, and only one extensive open reading frame (ORF), encoding *RNR4*, is detected within this region. To confirm that the mutated locus in *crt3-99* is allelic to *RNR4*, a *LEU2* marker was inserted next to the wild-type *RNR4* locus in a haploid by homologous integration and mated to the *crt3-99* haploid. From 12 tetrads dissected, the *LEU2* marker and the HU sensitivity always segregated exclusively in a 2:2 fashion, thus mapping the *crt3-99* mutation to the *RNR4* locus.

Plasmids. pMH120 was constructed by cloning the 3.1-kb *Nhe*I-*Pvu*II fragment from pMH78 into the *Spe*I-*Eco*RV sites of pBluescript KSII(−). pMH127, the *rnr4Δ::LEU2* disruption plasmid, was made by inserting *LEU2*-Km^r cassette between the *Stu*I and *Msc*I sites of *RNR4* in pMH120, thus replacing the entire coding region of *RNR4*. *Nhe*I-*Xho*I cleavage of pMH127 results in a 5.4-kb fragment for transplacement. pMH140 was made by cloning the 3.6-kb *Nhe*I-*Bam*HI fragment between the *Xba*I and *Bam*HI sites of pRS416 (*URA3*) (29).

The hemagglutinin (HA)- or Myc-tagged *RNR* gene expression constructs were made by creating convenient restriction enzyme sites outside the *RNR* ORFs by in vitro mutagenesis (Bio-Rad Mut-a-Gene kit), inserting an HA or a tandem triple Myc epitope-encoding sequence on double-stranded oligonucleotides at the amino termini, and subsequent subcloning into *CEN* vectors under the control of the *GAL* promoter. pMH172 and pMH178 contain Rnr2^{HA} and Rnr1^{HA} on a *TRP1* plasmid, respectively; pMH176, pMH180, and pMH195 contain Rnr4^{3XMyc}, Rnr1^{3XMyc}, and Rnr2^{3XMyc} on a *URA3* plasmid, respectively.

The construction of 2μm plasmids pBAD70, -71, -79, and -86 will be described elsewhere (4a). pBAD70 and pBAD71 are 2μm plasmids that contain the *RNR1* ORF under the control of the *GAP* promoter and contain *TRP1* and *URA3*, respectively. pBAD79 and pBAD86 are 2μm plasmids that contain the *RNR3* ORF under the control of the *GAP* promoter and contain *TRP1* and *HIS3* markers, respectively.

RNA blots. Total RNA was isolated by the hot acid-phenol method (16), resolved on formaldehyde–1% agarose gels, and hybridized to DNA probes as described previously (26).

Immunoprecipitation and Western blots. Yeast cells containing *GAL* expression plasmids were grown in SC-tryptophan-uracil containing 2% raffinose to an optical density at 600 nm of 0.4, induced with galactose at a final concentration of 2% (wt/vol) for 2 h, and harvested. Cell extracts were prepared by the glass bead disruption method as described previously (7). Yeast protein extracts (60 μg for each reaction) were diluted in 500 μl of immunoprecipitation buffer (25 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 μM aprotinin, 0.1 μg each of leupeptin, soybean trypsin inhibitor, pepstatin, and antipain per ml) and incubated with 5 μl of mouse anti-HA (12C5) or anti-Myc (9E10) monoclonal antibody (BAbCO) for 1 h at 4°C. The antibody-protein complexes were precipitated by absorption to protein A-Sepharose beads for 1 h at 4°C and washed twice with high-salt buffer (12 mM HEPES [pH 7.9], 500 mM NaCl, 2 mM EDTA, and 0.01% Tween 20) at room temperature. Western blot analysis was performed with the ECL system (Amersham).

Immunofluorescence staining and flow cytometry. Yeast cells expressing *GAL*-driven, Myc-tagged Rnr proteins were grown in SC-uracil with 2% raffinose as the carbon source to early log phase, induced by addition of galactose to 2%, and harvested 3 h later. Cells were fixed in 0.1 M potassium phosphate buffer (pH 6.5) with 4% formaldehyde at room temperature for 1 h and then digested with Zymolyase 100000 at 100 μg/ml in 0.1 M potassium phosphate buffer (pH 7.0)–1.2 M sorbitol at 37°C for 40 min. All of the following incubations were done at room temperature in phosphate-buffered saline–1% bovine serum albumin: first antibody (9E10) at a 1:50 dilution for 3 h, fluorescein isothiocyanate-conjugated secondary antibody at a 1:20 dilution for 1.5 h, and 1 μg of DAPI (4′,6-diamidino-2-phenylindole) per ml for 3 min. The flow cytometry (fluorescence-activated cell sorting [FACS]) assay was performed as described previously (1).

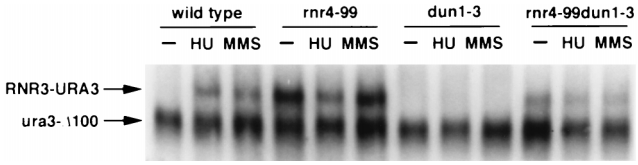

FIG. 1. Northern hybridization analysis of transcripts from *RNR3-URA3* in wild-type (Y203), *rnr4-99* (Y566), *dun1-3* (Y254), and *rnr4 dun1-3* (Y291) mutant strains. Individual cultures were grown to early log phase (optical density at 600 nm = 0.4) at 30°C and either kept growing without drug treatment (−) or treated with 150 mM HU or 0.01% MMS for 2 h at 30°C. Transcription from the *RNR3* promoter was visualized on Northern blots with a *URA3* probe. The higher-mobility band corresponds to the endogenous *ura3-Δ100* transcript; the lower-mobility band corresponds to the *URA3* transcript from the *RNR3* promoter.

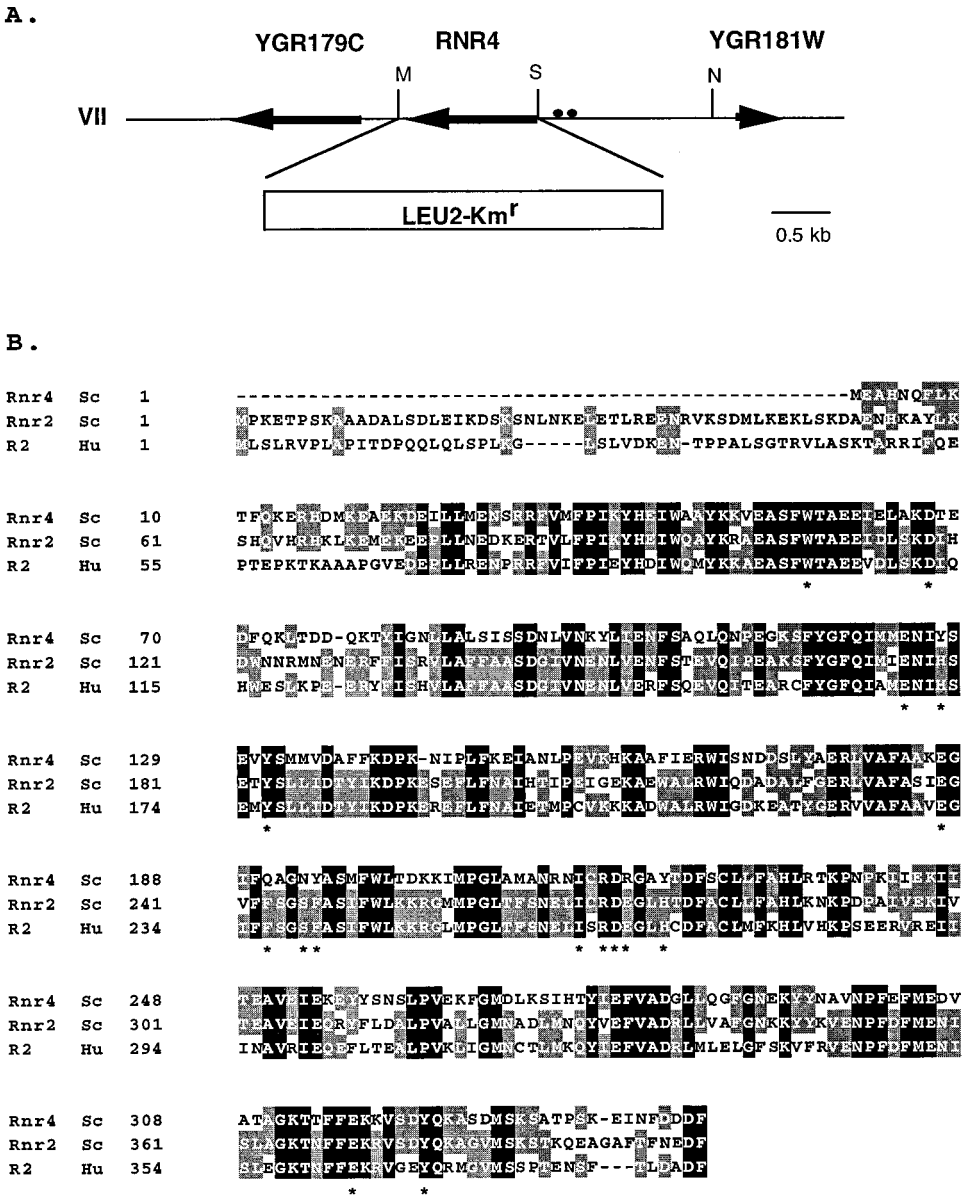

FIG. 2. *RNR4* locus and alignment between Rnr4, Rnr2, and human R2 protein. (A) Position of *RNR4* on chromosome VII. Arrows indicate the direction of transcription. *RNR4* is located between two ORFs, YGR179C and YGR181W. The two TATA-like sequences are indicated by dots. Restriction sites: N, *Nhe*I; S, *Stu*I, M, *Msc*I. The position of the replacement of the *RNR4* coding sequence with the *LEU2*-Km^r cassette is indicated. (B) Alignment between Rnr4, Rnr2, and human (Hu) R2 protein. Residues identical in all three proteins are in black boxes, and residues identical in only two proteins are in gray boxes. Asterisks in human R2 indicate the residues conserved in R2 proteins from mouse, clam, herpes simplex virus, Epstein-Barr virus, *E. coli*, and bacteriophage T4 (5). *Sc*, *S. cerevisiae.*

RESULTS

Identification of *RNR4* by complemention of *crt3-99*, a missense mutation that results in a Crt$^-$ phenotype. The *crt3-99* allele was identified in the screen for Crt$^-$ mutants. In addition to causing constitutive *RNR3* transcription, *crt3-99* mutants are also extremely sensitive to HU (38). To determine the epistasis between *CRT3* and *DUN1*, the signal-transducing kinase gene that controls the *RNR* gene transcriptional response after DNA damage (39), we made double mutants carrying *crt3-99* and *dun1-3*, an apparent null allele of *DUN1. crt3-99 dun1-3* double mutants exhibit an intermediate *RNR3* transcript level, higher than that of the wild type but lower than that of *crt3-99* single mutants (Fig. 1). Therefore, the *crt3-99* allele may act upstream of but not exclusively through *DUN1* to activate *RNR3* transcription.

We cloned the *CRT3* gene by complementation of both the Crt$^-$ phenotype and the HU sensitivity of the *crt3-99* mutant. The only extensive ORF within the 3.4-kb complementing fragment from chromosome VII was a gene with extensive homology to *RNR2* (Fig. 2). On this basis, we named this gene *RNR4*. We confirmed that the mutated locus in the *crt3-99* mutant is allelic to *RNR4* by linkage analysis with markers integrated at the *RNR4* locus (see Materials and Methods). Therefore, the gene and the mutation will be referred to as *RNR4* and *rnr4-99*, respectively. Sequence analysis of the *rnr4-99* allele revealed a GCA-to-GAA change at codon 184, replacing Ala with Glu.

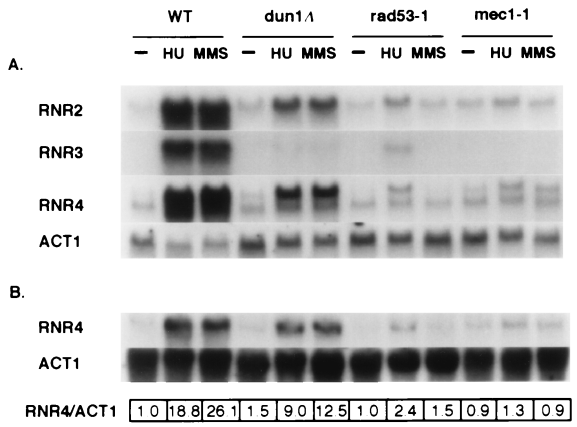

FIG. 3. Northern analyses of *RNR* genes. (A) Northern hybridization analysis of *RNR2*, *RNR3*, and *RNR4* in wild-type (WT) (TW397), *dun1-Δ100::HIS3* (Y567), *rad53-1* (TWY312), and *mec1-1* (TWY308) strains. Cells were grown to early log phase before being treated with 200 mM HU or 0.01% MMS for 2 h at 30°C. −, RNA from untreated cells. mRNAs were detected by different *RNR* gene probes and the *ACT1* probe. (B) Northern blot of the same samples as in panel A probed with the 118-bp fragment between the two TATA-like sequences in the 5′ untranslated region of *RNR4*. The numbers at the bottom of each lane indicate the relative intensity of the *RNR4* signal, normalized against the *ACT1* signal.

The *RNR4* ORF encodes a predicted polypeptide of 345 amino acids. *RNR2* and *RNR4* have extensive sequence identity in the coding region, 56% at the amino acid level and 69% at the nucleic acid level (Fig. 2). However, the *RNR4* product is about 50 amino acids shorter than that of *RNR2* and than other mammalian small subunits at the N terminus. Another unusual feature of the *RNR4* product is that it diverges from the other known R2 sequences in many of the conserved residues, with 6 of the 16 residues that are absolutely conserved from *E. coli* to mammals (5) changed. The most striking change is that the His residue (amino acid 127) that is coordinated to the iron center required for catalytic activity is replaced by a Tyr in Rnr4. A search of the *S. cerevisiae* gene bank confirmed that *RNR4* and *RNR2* are the only two yeast genes encoding the RNR small subunits and that no other class II- or class III-related reductases appear to exist.

The *RNR4* gene has two transcripts, one of which is highly inducible by DNA damage and replication blocks. One common feature of the known *RNR* genes is that their transcription is upregulated upon DNA damage or replication blocks. We tested whether *RNR4* is also DNA damage inducible by Northern blot analysis. Two transcripts of about 1 kb were detected by a probe covering the full-length *RNR4* ORF. The longer transcript is induced both by the alkylating reagent methyl methanesulfonate (MMS) and by HU, whereas the shorter transcript is not (Fig. 3A). Neither of the *RNR4* transcripts shows obvious fluctuation during the cell cycle (data not shown). Examination of the promoter region of *RNR4* revealed two TATA-like sequences near the translational start (data not shown), suggesting the existence of two different transcriptional initiation sites. A probe corresponding to the 118-bp sequence between the two TATA-like sequences detected only the longer, DNA damage-inducible transcript on Northern blotting (Fig. 3B). Therefore, it is likely that the more-upstream TATA box gives rise to the longer transcript and that the more-downstream TATA box gives rise to the shorter transcript. The function of two differentially regulated *RNR4* transcripts is unclear, since they should encode the same protein. It is interesting that the *Schizosaccharomyces pombe* RNR small-subunit gene *suc22*$^+$ also gives rise to two transcripts, and only the longer transcript is induced by HU (13, 14).

DNA damage inducibility of the *RNR* genes is controlled by the signal-transducing kinase genes *MEC1*, *RAD53*, and *DUN1*. The DNA damage signal transduction pathway in yeast includes three known kinase genes as transducers, the central transducer genes *MEC1* and *RAD53*, which control both cell cycle arrest and transcriptional response, and *DUN1*, which is involved only in the transcriptional response (1, 27, 36, 39). The DNA inducibility of *RNR2*, *RNR3*, and *RNR4* in wild-type and *mec1-1*, *rad53-1*, and *dun1* null mutants was examined. The DNA damage inducibility of all three *RNR* genes is reduced in *dun1* null mutants and almost abolished in *rad53-1* and *mec1-1* mutants (Fig. 3A). The minor increase of *RNR* levels in *rad53-1* mutants treated with HU may be due to S-phase synchronization of the cell population or residual wild-type Rad53 activity in *rad53-1* mutants.

***rnr4-99* delays S-phase progression and is synthetically lethal with mutant alleles of *DUN1*, *RAD53*, and *MEC1*.** Northern analysis showed that the *RNR3* level in *rnr4-99 dun1-3* double mutants is much higher than that in wild-type cells although lower than that in *rnr4-99* single mutant cells (Fig. 1). Since *dun1-3* is an apparent null allele of *DUN1* (39), these results indicate that there is a *DUN1*-independent branch for *RNR3* induction. The transcript levels of *RNR2* and *RNR4* also show some increase upon HU and MMS treatment in *DUN1* deletion mutants (Fig. 3A), suggesting that a pathway independent of *DUN1* is involved in the DNA damage-induced transcription of *RNR* genes. To exclude the possibility of residual *DUN1* activity in *dun1-3* mutants, we attempted to make an *rnr4-99 dun1-Δ100::HIS3* double mutant. No nonparental ditype tetrads with more than two viable spores and no tetratype tetrads with four viable spores were recovered, suggesting that *rnr4-99 dun1-Δ100::HIS* is lethal. The synthetic lethality was confirmed by the failure of *rnr4-99 dun1Δ* mutants carrying *RNR4* on a *URA3* plasmid to grow on 5-FOA plates, apparently due to the lethal event of losing the wild-type *RNR4* copy. Similar analyses revealed that *rnr4-99* is also synthetically lethal with *rad53-21* and *mec1-21* alleles (Fig. 4A). *rnr4-99*, like mutations in *RNR1* and *RNR2* (38), is likely to lie upstream of damage signal transducer genes by causing dNTP pool depletion and generating a constitutive replication-blocking signal, which cannot be properly relayed in *mec1-21*, *rad53-21*, or *dun1Δ* mutants. FACS analysis revealed that *rnr4-99* mutant cells in mid-log-phase culture accumulate in S phase (Fig. 4B). When released from G_1 arrest by α-factor, *rnr4-99* mutant cells exhibit a 30-min delay in exit from S phase compared to wild-type cells (Fig. 4B). These findings are consistent with the idea that *rnr4-99* mutants are defective in DNA synthesis.

***RNR2* and *RNR4* are both essential for mitotic viability.** To determine the null phenotype of *RNR4* mutants, an *rnr4Δ::LEU2* deletion allele (Fig. 2A) was introduced into a diploid strain, Y323, by transplacement (25). Disruptions were verified by Southern blot analysis. Tetrad analysis was performed with two independently derived diploids heterozygous for an *rnr4Δ::LEU2* disruption. None of the 26 tetrads analyzed gave rise to more than two viable spores, and all of the viable spores were Leu$^-$ auxotrophs (Table 2). The inviable spores did germinate and undergo one or two rounds of cell division before arrest as cells with elongated buds. Therefore, *RNR4* is essential for mitotic viability.

RNR2 has been shown from previous studies to be essential for mitotic viability, although in strains different from Y323 (5). *rnr2Δ::TRP1* disruptants were made from Y323 in the same manner as described previously (5), and a similar terminally arrested phenotype was observed for *rnr2Δ::TRP1* hap-

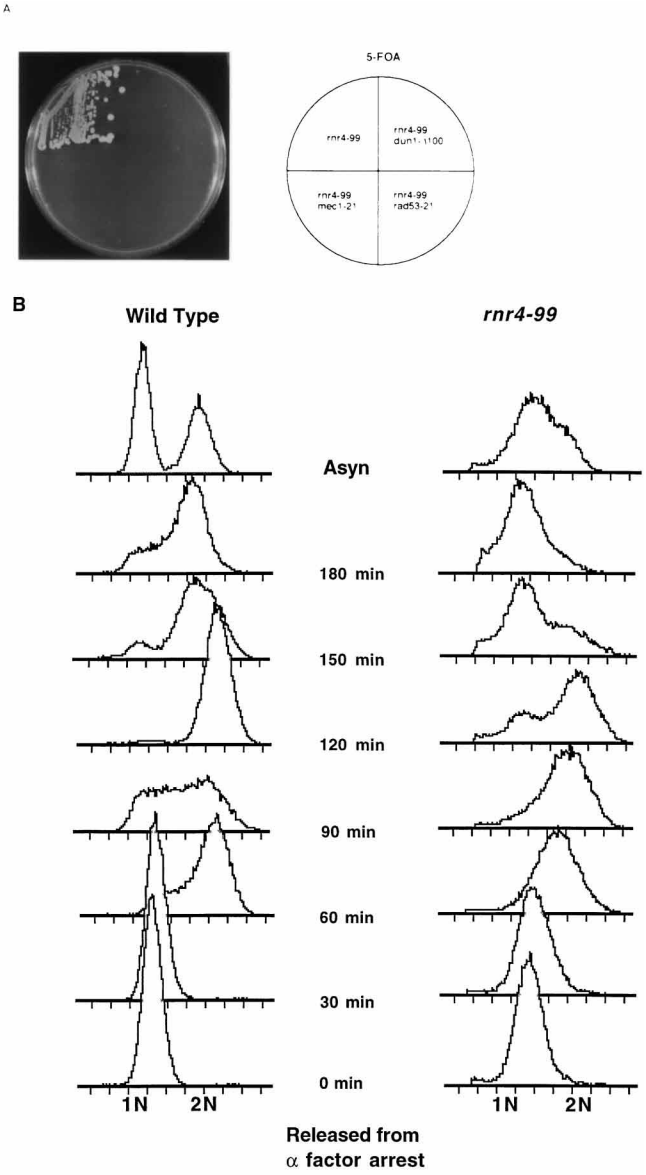

FIG. 4. *rnr4-99* mutants exhibits a delay in S-phase progression, and *rnr4-99* is synthetically lethal with mutant alleles of *dun1*, *rad53*, and *mec1*. (A) *rnr4-99* is synthetically lethal with *dun1-Δ100::HIS3*, *rad53-21*, and *mec1-21*. All strains shown carry the plasmid pMH140 (*URA3 RNR4*) and were streaked onto a 5-FOA plate. Failure to grow on 5-FOA plates indicates that the lethality resulted from the loss of the wild-type *RNR4* copy on the *URA3* plasmid. The strains are Y568 (*rnr4-99*), Y569 (*rnr4-99 dun1-Δ100::HIS3*), Y570 (*rnr4-99 rad53-21*), and Y571 (*rnr4-99 mec1-21*). (B) FACS profiles of wild-type (Y300) and *rnr4-99* (Y568) cells from asynchronously grown cultures (Asyn) and cells at different time points after release from α-factor-induced G_1 arrest.

loids. Thus, both *RNR2* and *RNR4* are essential for mitotic viability. The essentiality of either small-subunit gene cannot be complemented by the presence of multiple copies of the other, suggesting that each has a unique function (data not shown).

***RNR4* and *RNR2* may be present in the same reductase complex in vivo.** The finding that *RNR2* and *RNR4* are both essential suggests that there may be several functional isoforms of RNR in vivo, such as $\alpha_2\beta_2$, $\alpha_2\beta'_2$, and $\alpha_2\beta\beta'$ (α = *RNR1*, β = *RNR2*, and β' = *RNR4*). These isoforms may have different specificities or localizations. To determine if *RNR2* and *RNR4* can form a complex in vivo, coexpression of *GAL*-driven, epitope-tagged *RNR2* and *RNR4* was induced by addition of galactose and immunoprecipitation was performed. $Rnr2^{HA}$ was detected in anti-Myc immunoprecipitates of $Rnr4^{3XMyc}$ by immunoblotting with anti-HA (Fig. 5C, lane 5). $Rnr2^{HA}$ was not coimmunoprecipitated with anti-Myc in extracts from cells not expressing $Rnr4^{3XMyc}$ (Fig. 5C, lane 4). However, little $Rnr4^{3XMyc}$ was detected in immunoprecipitates of $Rnr2^{HA}$ (Fig. 5C, lane 8). It is possible that anti-HA binding to $Rnr2^{HA}$ interferes with Rnr2-Rnr4 interaction or that Rnr4 binding to $Rnr2^{HA}$ obscures the HA epitope. We also tested the possible interactions between Rnr1 and Rnr2 or Rnr4. Although both $Rnr1^{3XMyc}$ and $Rnr4^{3XMyc}$ can be immunoprecipitated by anti-Myc antibody (Fig. 5C, lanes 10 and 12), no $Rnr2^{HA}$ (lane 4) or $Rnr1^{HA}$ (lane 6) was detected in the immunoprecipitates. $Rnr1^{HA}$ was not immunoprecipitated with anti-HA antibody under the same conditions (Fig. 5C, lane 3). The levels of *GAL*-driven *RNR2* and *RNR4* expression are comparable to those of wild-type cells upon DNA damage or a replication block (Fig. 5A, lanes 2, 3, and 5) and are therefore within the normal physiological range of *RNR* levels.

TABLE 2. Tetrad analysis of the *RNR4/rnr4Δ-127* and *RNR2/rnr2Δ-312* diploid strains Y572 and Y573

Strain	No. of viable spores per tetrad	No. of tetrads observed	Genotype	
			trp$^-$	*TRP*$^+$
Y572	4	0	0	0
	3	0	0	0
	2	26	52	0
	1	2	2	0
Y573			*ura*$^-$	*URA*$^+$
	4	0	0	0
	3	0	0	0
	2	25	50	0
	1	3	3	0

The lethality of *RNR4* deletion can be suppressed by overexpression of the large-subunit genes *RNR1* and *RNR3*. It is unclear which subunit(s) of the RNR is rate-limiting for enzyme complex assembly. One possible mechanism for the lethality of deletion of either *RNR2* or *RNR4* is that the mutant cells have a lower level of functional RNR complex, resulting in dNTP levels below the threshold critical for mitotic viability. We tested whether increasing the level of the large-subunit genes can suppress the lethality caused by deficiency in the small-subunit genes by plasmid shuffle and tetrad analyses. Both *RNR1* and *RNR3* overexpression can suppress *rnr4Δ* lethality (Fig. 6), but neither can suppress *rnr2Δ* lethality (data not shown). *RNR1* overexpression also suppressed the lethality of *rnr4-99 dun1-Δ100::HIS3* double mutants (data not shown).

Subcellular localization of the large and small subunits of the RNR enzymes. The subcellular localization of the RNR subunits was examined by immunofluorescence studies of the *GAL*-driven, Myc_3-tagged Rnr proteins. Both $Rnr1^{3XMyc}$ and $Rnr2^{3XMyc}$ showed stronger staining in the cytoplasm than in the nucleus, whereas $Rnr4^{3XMyc}$ showed stronger staining in the nucleus (Fig. 7). No apparent change in the staining pattern was observed upon DNA damage or replication blocks (data not shown).

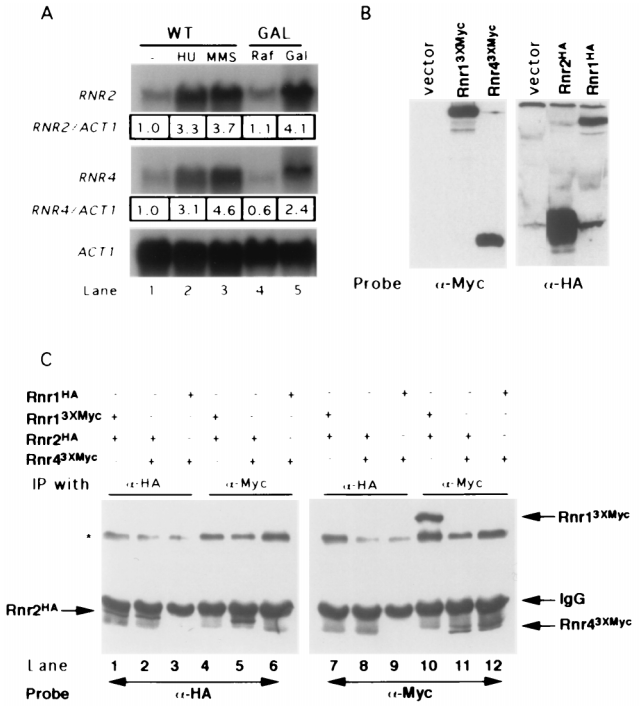

FIG. 5. Rnr4 and Rnr2 form a complex in vivo. (A) Northern analysis of endogenous and *GAL*-driven *RNR2* and *RNR4* transcription. Wild-type cells (WT) (Y300) were grown to early log phase, kept untreated (−) or treated with 200 mM HU or 0.01% MMS for 2 h at 30°C, and harvested for RNA extraction. Wild-type cells carrying *GAL*-driven *RNR2* (pMH172) and *RNR4* (pMH176) plasmids were grown in SC-tryptophan-uracil medium plus 2% raffinose (Raf) to early log phase, induced by addition of galactose to 2% and growth for 2 h at 30°C (Gal), and harvested for preparation of RNA. The numbers at the bottom of each lane indicate the relative abundances of *RNR2* and *RNR4*, normalized against *ACT1*. (B) Western analysis of *GAL*-driven $Rnr1^{3XMyc}$ (pMH180), $Rnr1^{HA}$ (pMH178), $Rnr2^{HA}$ (pMH172), and $Rnr4^{3XMyc}$ (pMH176). Cells were treated as described for panel A, and 60 μg of protein extract was loaded in each lane. There are two cross-reacting bands in the anti-HA (α-HA) blot, one near the position of the $Rnr2^{HA}$ band and the other above the position of the $Rnr1^{HA}$ band. (C) Immunoprecipitation (IP) of Rnr proteins. Sixty micrograms of extract from a wild-type strain (Y300) that contains two plasmids expressing HA- and Myc-tagged Rnr proteins were immunoprecipitated with α-HA (lanes 1 to 3 and 7 to 9) or α-Myc (lanes 4 to 6 and 10 to 12) and then immunoblotted with α-HA (lanes 1 to 6) or α-Myc (lanes 6 to 12). IgG, heavy chain of immunoglobulin G. The asterisk indicates an unknown protein that cross-reacts with the secondary antibody.

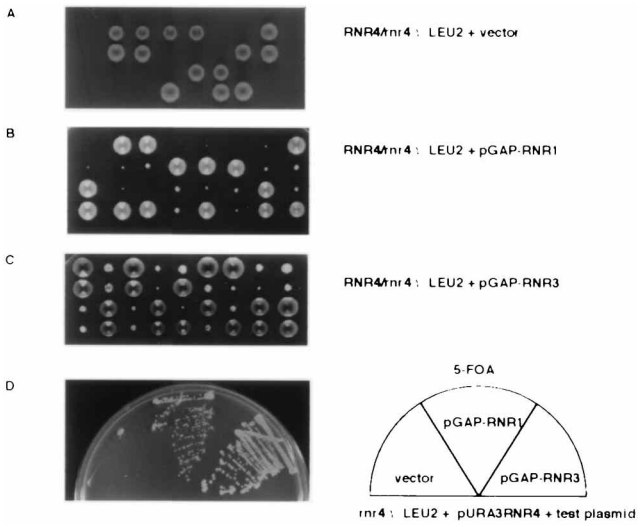

FIG. 6. Overexpression of *RNR1* and *RNR3* suppresses the lethality of *RNR4* deletion mutations. *rnr4Δ::LEU2/RNR4*, pMH140 (*URA3 RNR4*) diploid strains containing vector alone (pBAD52, Y576) (A), a plasmid expressing *RNR1* (pBAD70, Y574) (B), and a plasmid expressing *RNR3* (pBAD79, Y575) from the *GAP* promoter (C) were sporulated. Tetrads were dissected, and the spores were incubated at 30°C for 3 to 4 days. (D) Plasmid shuffle analysis. *rnr4Δ::LEU2*, pMH140 haploid strains carrying pBAD52, pBAD70, or pBAD79 were grown on 5-FOA plates. Growth on 5-FOA plates indicates that the *rnr4Δ* strain can survive in the absence of the wild-type copy of *RNR4* on the *URA3* plasmid pMH140.

DISCUSSION

Why are there two essential RNR small-subunit genes in yeast? The RNR enzymes from both prokaryotic and eukaryotic organisms have long been known to have an $\alpha_2\beta_2$ protein structure, consisting of two large (R1) and two small (R2) subunits (reviewed in reference 23). In the budding yeast, the large subunit is encoded by two genes, *RNR1* and *RNR3*. Under normal vegetative conditions, the enzyme is thought to be mostly of the $\alpha 1_2\beta_2$ form, since only *RNR1* transcript is detectable. Upon DNA damage, *RNR3* is highly induced, and thus the enzyme may have two more isoforms, $\alpha'_2\beta_2$ and $\alpha\alpha'\beta_2$ (8). The small subunit of RNR contains the tyrosyl free radical coordinated with a binuclear iron center that is essential for enzymatic activity. *RNR2* is an essential gene that encodes this small subunit in *S. cerevisiae*. In this paper we have described the identification of a second essential gene encoding the small subunit of RNR, *RNR4*. In the accompanying paper (35) Wang et al. describe the independent isolation of the *RNR4* gene and show that *rnr4* null mutants are viable but sensitive to both high and low temperatures. The difference is likely to be due to the different genetic backgrounds of the stains used in the two studies, because we have used our replacement construct in their strain and obtained results similar to theirs (15a).

There are several plausible explanations for yeast cells having two essential RNR small-subunit genes. (i) The level of *RNR2* fluctuates by about twofold during cell cycle (8), whereas that of *RNR4* does not. Thus, *RNR4* may compensate for *RNR2* activity when *RNR2* is low outside S phase. The evidence against this explanation is that a plasmid constitutively overexpressing *RNR2* (pMH172 *GAL*-$Rnr2^{HA}$) can complement *rnr2* mutants but can not rescue *rnr4* deletion mutants. (ii) It is possible that the small subunits also contribute to the regulation of the enzyme so that different RNR isoforms have different substrate specificities and enzymatic activities in vivo. Therefore, disruption of either *RNR2* or *RNR4* may perturb the balance of the dNTP pools, which is detrimental to mitotic viability. It is important to note that while overexpression of *RNR1* suppressed *rnr4Δ* lethality, *RNR3* levels are likely to be high as well. This leads to the possibility that reductases of the composition $\alpha\alpha'\beta_2$, $\alpha_2\beta_2$, and $\alpha'_2\beta_2$ exist under these circumstances and contribute to viability. (iii) It is possible that RNR in yeast is generally of the form $\alpha_2\beta\beta'$ and that enzymes of the form $\alpha_2\beta_2$ and $\alpha_2\beta_2'$ are either inactive or assemble poorly. (iv) Since Rnr2 and Rnr4 may be partially differentially localized inside the cell, with Rnr2 mostly in the cytoplasm and Rnr4 mostly in the nucleus, it is possible that they each play essential roles in different subcellular compartments. One caveat is that the immunofluorescence analyses were performed with *GAL*-driven, epitope-tagged Rnr proteins and therefore may not represent the localization of the endogenous protein. For example, Rnr1 may require association with a limiting factor, e.g., Rnr4, to enter the nucleus, and overproduction could give artifactual results.

One intriguing feature of *RNR4* is that its product has sev-

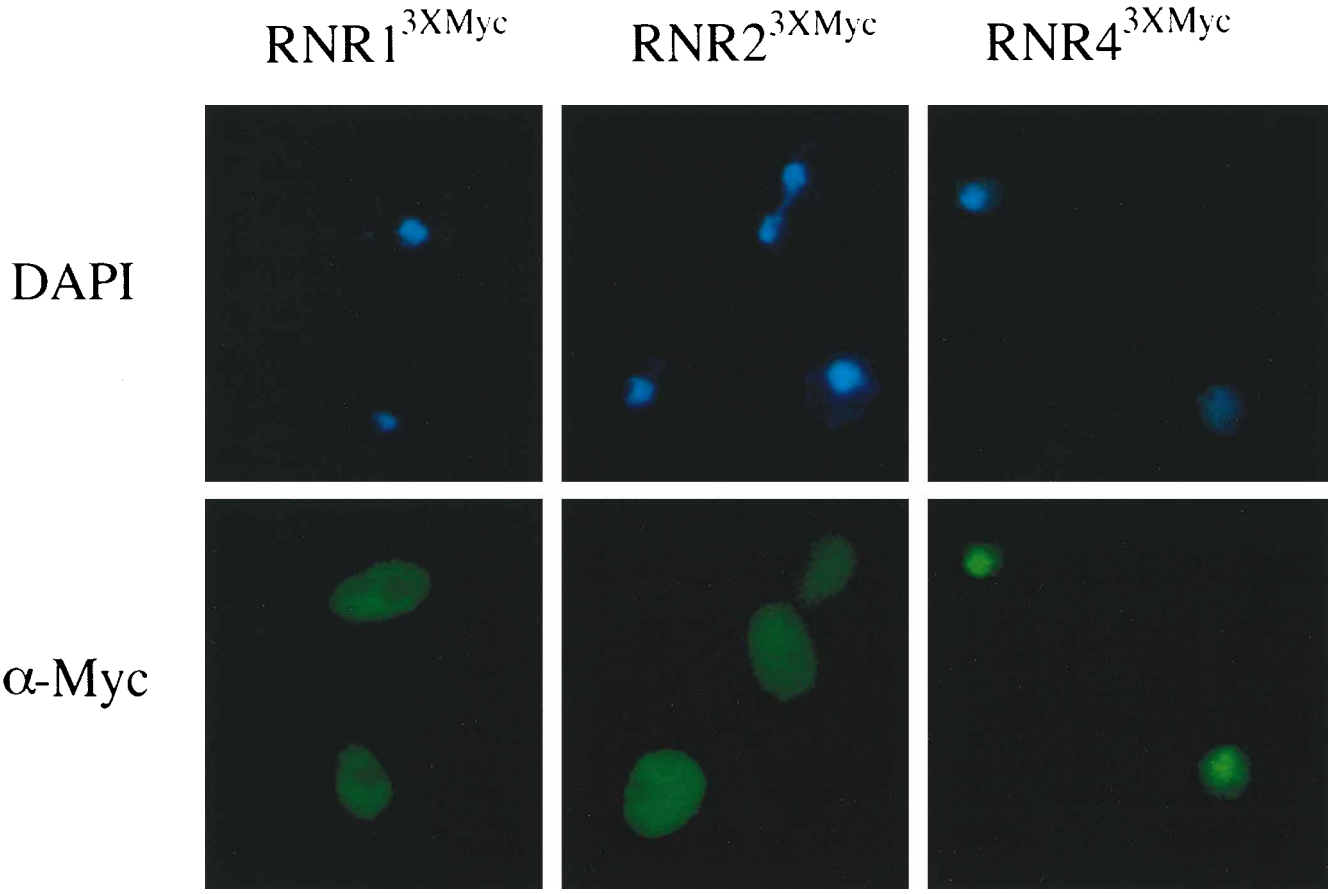

FIG. 7. Immunofluorescence staining of Myc-tagged Rnr1, Rnr2, and Rnr4. Y300 containing *GAL*-driven Rnr1^{3XMyc} (pMH180), Rnr2^{3XMyc} (pMH195), or Rnr4^{3XMyc} (pMH176) was grown in SC-uracil containing 2% raffinose and was induced for 3 h with 2% galactose prior to fixing and staining as described in Materials and Methods.

eral changes in residues that are otherwise completely conserved among R2 subunits through evolution. One example is that the conserved His (residue 127) that is coordinated to the iron center in R2 and essential for catalytic activity is replaced by a tyrosine in the *RNR4* product. These changes raise the question as to whether the *RNR4* product acts as a functional tyrosol free radical provider in vivo. We feel that given these changes, it is highly likely that the *RNR4* product is not catalytically active and is likely to act in a structural capacity. In support of this, Wang et al. (35) found that substitution of Phe for the conserved Tyr (residue 131), which generates the free radical in the R2 subunit, still complements the *rnr4* deletion mutants. This indicates that Rnr4 need not be catalytically active to carry out its essential function. However, since *rnr4-99* mutants exhibit a delay in S-phase progression and are very sensitive to HU, *RNR4*'s role is likely to involve dNTP synthesis and is likely to act in support of *RNR2* and *RNR1* activity. In further support of this, Wang et al. (35) found that the endogenous levels of Rnr activity in vitro were very low but could be significantly increased by addition of bacterially expressed Rnr1 protein. However, extracts from *rnr4* mutants supplemented with Rnr1 reached only 15% of the wild-type RNR activity levels, indicating a defect in Rnr function. Addition of bacterially expressed Rnr4 to these extracts could restore full RNR activity. These results, together with our observations that Rnr2 and Rnr4 physically associate, suggest that Rnr4 is a structural component of the RNR enzyme. It may play a structural or a regulatory nonenzymatic role in enzyme activity.

Why does *RNR1* and *RNR3* overexpression suppress the lethality of an *rnr4* deletion? The RNR enzyme activity requires both large and small subunits. It is unclear how the formation of functional RNR enzyme is regulated. The finding that overexpression of the large subunit can suppress the lethality of an *rnr4* deletion mutation suggests that the large subunit may be the rate-limiting step in assembly of functional RNR enzyme in vivo. The data from Wang et al. have shown that the large subunit is rate-limiting in vitro (35). This is consistent with the fact that *RNR1* mRNA is very tightly cell cycle regulated (8). *rnr4* null mutants may have a lower-than-critical concentration of either certain dNTPs globally or all dNTPs regionally (e.g., near DNA polymerases). An increase in the level of the large subunit forces the assembly of the enzyme complex, perhaps in an $\alpha_2\beta_2$ configuration, and compensates for the decrease of dNTP levels due to *RNR4* loss. However, overexpression of either *RNR1* or *RNR3* cannot suppress *rnr2* null mutations. It is possible that *rnr2* deletion makes the level of the small subunit become the rate-limiting factor for RNR assembly, which cannot be compensated for by an increase in the large subunit. Alternatively, perhaps only Rnr2 is catalytically active and Rnr4 is inactive. Since *RNR4* and *RNR2* coimmunoprecipitate, we feel that they are likely to exist as a $\beta\beta'$ dimer in which β (Rnr2) has catalytic activity and β' (Rnr4) plays a largely structural role. Thus, the yeast enzyme is

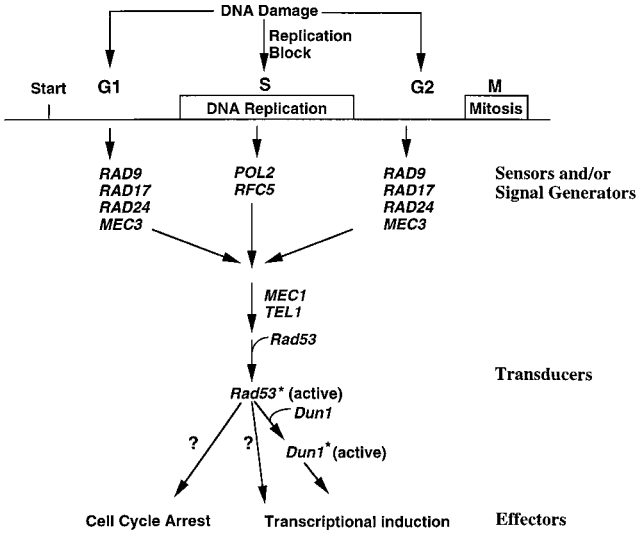

FIG. 8. Model for DNA damage signal transduction pathway in yeast. Sensors of DNA damage signals in G_1 and G_2-M phases are *Rad9*, *Rad17*, *Rad24*, and *Mec3* (19). Putative sensors of DNA damage and replication blocks during S phase are *Pol2* (21) and *Rfc5* (32). The signal generated by these sensors is transduced through kinases *Mec1* and *Tel1*, leading to phosphorylation and activation of Rad53 (marked as Rad53*) (27, 34). Rad53* causes cell cycle arrest and transcriptional induction. The transcriptional response can be mediated by two mechanisms, one through activation of Dun1 kinase (marked as Dun1*) (1) and the other through a *DUN1*-independent mechanism.

likely to be of the structure $\alpha_2\beta\beta'$, a structure unique among RNRs.

A *DUN1*-independent pathway for *RNR* transcriptional induction upon DNA damage. Mutations in three *RNR* genes, *RNR1* (*CRT7*), *RNR2* (*CRT6*), and *RNR4* (*CRT3*) have been isolated in the Crt^- screen (38). These mutations, like mutations in other genes involved in dNTP metabolism, are likely to lead to an endogenous DNA damage or replication block signal due to dNTP pool depletion, which activates the DNA damage signaling pathway resulting in a constitutive transcriptional response, the Crt^- phenotype. Epistasis analysis suggests that *rnr4-99* acts partially but not completely through Dun1 kinase, since the *RNR3* level in *rnr4-99 dun1-3* double mutants is intermediate between those in *rnr4-99* mutants and the wild type. Northern analysis shows that there is still significant *RNR2* and *RNR4* induction after HU and MMS treatment in the *dun1* null mutant (Fig. 3A), and this induction is further decreased in *mec1* and *rad53* mutants. These data indicate that the regulation of *RNR2* and *RNR4* transcription is not completely controlled by Dun1, as illustrated in the model in Fig. 8.

Therefore, there is a *DUN1*-independent mechanism for *RNR2* and *RNR4* regulation in response to DNA damage. Perhaps there exists a gene that carries out *DUN1*'s function. There is a *DUN1*-related gene, *MRE4*/*MEK1*, which is structurally similar to *DUN1* both in the kinase domain and in the regulatory region, where both have an FHA domain (15). However, *MRE4* is meiosis specific (17, 24) and is therefore unlikely to perform a *DUN1*-redundant role. Rad53 also contains a forkhead-associated (FHA) domain and is closely related to Dun1 in the kinase domain, both being of the calmodulin-dependent kinase protein kinase subfamily (30, 39). It is possible that Rad53 itself can activate *RNR* transcription by two mechanisms, one indirectly by activating Dun1 (1) and one directly either by recognizing Dun1 substrates or through a completely distinct mechanism. There is some evidence for redundant function between *DUN1* and *RAD53*, because a *rad53-21* mutation is lethal in combination with a deletion of *DUN1* (15b). It remains to be seen what constitutes this parallel pathway. However, it is clear that the transcriptional response to DNA damage is more complex than initially perceived and will likely involve additional genes that have yet to be implicated in this response.

ACKNOWLEDGMENTS

We thank Z. Zhou and members of the Elledge lab for helpful discussions and/or reagents, and we thank Tim Huffaker for sharing results prior to publication.

This work was supported by grant GM44664 from the National Institutes of Health and grant Q1187 from the Robert A. Welch Foundation to S.J.E. M.H. was a Robert A. Welch postdoctoral fellow and is currently supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Foundation. S.J.E. is a PEW Scholar in the Biomedical Sciences and an Investigator of the Howard Hughes Medical Institute.

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