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Rnr4p, a Novel Ribonucleotide Reductase Small-Subunit Protein

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Ribonucleotide reductases catalyze the formation of deoxyribonucleotides by the reduction of the corresponding ribonucleotides. Eukaryotic ribonucleotide reductases are $\alpha_2\beta_2$ tetramers; each of the larger, α subunits possesses binding sites for substrate and allosteric effectors, and each of the smaller, β subunits contains a binuclear iron complex. The iron complex interacts with a specific tyrosine residue to form a tyrosyl free radical which is essential for activity. Previous work has identified two genes in the yeast *Saccharomyces cerevisiae*, *RNR1* and *RNR3*, that encode α subunits and one gene, *RNR2*, that encodes a β subunit. Here we report the identification of a second gene from this yeast, *RNR4*, that encodes a protein with significant similarity to the β-subunit proteins. The phenotype of *rnr4* mutants is consistent with that expected for a defect in ribonucleotide reductase; *rnr4* mutants are supersensitive to the ribonucleotide reductase inhibitor hydroxyurea and display an S-phase arrest at their restrictive temperature. *rnr4* mutant extracts are deficient in ribonucleotide reductase activity, and this deficiency can be remedied by the addition of exogenous Rnr4p. As is the case for the other *RNR* genes, *RNR4* is induced by agents that damage DNA. However, Rnr4p lacks a number of sequence elements thought to be essential for iron binding, and mutation of the critical tyrosine residue does not affect Rnr4p function. These results suggest that Rnr4p is catalytically inactive but, nonetheless, does play a role in the ribonucleotide reductase complex.

Ribonucleotide reductases catalyze the formation of deoxyribonucleotides by the reduction of the corresponding ribonucleotides. Three classes of ribonucleotide reductases have been well characterized (24). Class I enzymes are found in all eukaryotes and some prokaryotes. The best-studied class I enzyme is the *Escherichia coli* ribonucleotide reductase (10, 30), an $\alpha_2\beta_2$ tetramer that can be decomposed to two catalytically inactive homodimers, R1 (α_2) and R2 (β_2). Each of the larger α subunits possesses binding sites for substrate and allosteric effectors and also contains several redox-active cysteine residues. Each of the smaller β subunits contains a binuclear Fe(III) complex. The X-ray structure of *E. coli* R2 reveals that the iron ions are bridged by both an O^{2-} ion and the carboxyl group of a glutamate residue (22). Each iron is further liganded by two carboxyl oxygen atoms from aspartate or glutamate residues, a histidine Nδ residue, and a water molecule. The recently solved structure of the mouse R2 protein indicates that the iron-binding center of eukaryotic proteins is similar to that of the *E. coli* protein (17). The iron complex interacts with a specific tyrosine residue to form a tyrosyl free radical which is essential for activity. The enzyme is inhibited by hydroxyurea, which specifically quenches the tyrosyl radical (19).

Amino acid sequence alignments of the class 1 R2 proteins from different species identify 16 residues that are conserved in all of these proteins reported to date (7, 22). Most of these conserved residues are at the iron center or close to it.

Previous work has identified two genes in the yeast *Saccharomyces cerevisiae* (*RNR1* and *RNR3*) that encode R1 proteins (8) and one gene (*RNR2*) that encodes an R2 protein (7, 13). Here we report the identification of a second gene from this yeast, *RNR4*, that encodes a protein with significant similarity to the R2 proteins. However, Rnr4p lacks a number of sequence elements thought to be essential for enzymatic function. Our evidence suggests that Rnr4p is catalytically inactive but, nonetheless, does play a role in the ribonucleotide reductase complex.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used for these experiments are listed in Table 1. A yeast strain containing the *qcr9-Δ1* mutation was obtained from B. Trumpower (Dartmouth College, Hanover, N.H.) and backcrossed to CUY8 to obtain CUY977. A yeast strain containing the *rnr2-68* mutation was obtained from S. Elledge (Baylor College of Medicine, Houston, Tex.) and backcrossed to CUY8 to obtain CUY989 and CUY990. DL2 and DL2rho^0 were obtained from T. Fox (Cornell University, Ithaca, N.Y.).

Yeast extract-peptone-dextrose (YPD), yeast extract-peptone-glycerol (YPG), and synthetic minimal (SD) media and plates were prepared as described by Sherman (28). Sporulation medium was 0.3% potassium acetate. Plates containing hydroxyurea or benomyl were made by adding the indicated amount of these drugs to YPD agar after autoclaving.

Cloning and disruption of *RNR4*. The *RNR4* gene was cloned by complementation of the *rnr4-1* cold-sensitive phenotype. CUY58 was transformed with a yeast genomic library constructed in the *URA3* YCp50 vector (26). Following transformation, cells were plated on SD lacking uracil, grown at 30°C for 16 h, and then shifted to 14°C. One Ura$^+$ transformant which grew at 14°C was isolated. The plasmid recovered from this strain, pRC1, was able to complement the cold-sensitive phenotype of *rnr4-1* following retransformation back into CUY58.

The *RNR4* gene was disrupted by the one-step gene replacement method (27). The 3-kb *Xba*I-*Bam*HI fragment of pRC1 was subcloned into the *Spe*I-*Bcl*I sites of pCR2.1 (Invitrogen Corp., Carlsbad, Calif.). The 950-bp *Bgl*II-*Eco*RI fragment was then replaced with a 3-kb *Bam*HI-*Eco*RI fragment containing the *HIS3* gene to create pWP77. This construct deletes *RNR4* sequences extending from 50 bp upstream of the start codon to codon 302. The 4.2-kb *Spe*I-*Spe*I fragment of pWP77 was gel purified and transformed into the diploid strain CUY546. Two transformants were isolated. Southern blotting and PCR confirmed that in each case one copy of *RNR4* was disrupted by *HIS3* (data not shown). Sporulation and

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TABLE 1. Yeast strains

Strain	Genotype
CUY8	*MAT***a** *ade2-101 ura3-52*
CUY25	*MAT***a** *ade2 his3-Δ200 leu2-3,112 ura3-52*
CUY26	*MATα his3-Δ200 leu2-3,112 ura3-52*
CUY58	*MAT***a** *rnr4-1 his4 ura3-52*
CUY59	*MATα rnr4-1 his4 ura3-52*
CUY111	*MATα TUB3::URA3::TUB3 his3-Δ200 leu2-3,112 trp1-1 lys2-801 ura3-52*
CUY117	*MATα TUB1::URA3::ΔTUB1 his3-Δ200 leu2-3,112 lys2-801 ura3-52*
CUY546	*MAT***a**/*MATα ade2/ADE2 his3-Δ200/his3-Δ200 leu2-3, 112/leu2-3,112 ura3-52/ura3-52*
CUY848	*MATα tub2-469::URA3 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52*
CUY977	*MAT***a** *qcr9-Δ1::URA3*
CUY989	*MATα rnr2-68 his3 leu2-3,112 lys2 trp1 ura3*
CUY990	*MAT***a** *rnr2-68 his3 leu2-3,112 lys2 trp1 ura3*
CUY995	*MATα rnr4-Δ1::HIS3 ade2-101 leu2-3,112 ura3-52*
CUY996	*MAT***a** *rnr4-Δ1::HIS3 leu2-3,112 ura3-52*
DL2	*MAT***a** *lys2*
DL2rho^0	*MAT***a** *lys2 rho^0*

tetrad dissection were used to obtain haploid cells containing the disruption (*rnr4-Δ1*). *rnr4-Δ1* cells from the two transformants behaved similarly.

Plasmid constructs. The 2.5-kb *Kpn*I-*Bam*HI fragment of pRC1 (see Fig. 1) was subcloned into pRS416 (29) to create pRC2, a YCp plasmid containing *RNR4*. The 2.5-kb *Kpn*I-*Bam*HI fragment of pRC1 was also subcloned into pRS426 (4) to create pRC4, a YEp plasmid containing *RNR4*. pSE327, a YEp plasmid containing RNR2, was obtained from S. Elledge (Baylor College of Medicine).

The *rnr4-2* allele was constructed by oligonucleotide-directed mutagenesis by the overlapping PCR method as described by Eichinger et al. (6). Four oligonucleotide primers were used; primer A (AAAAATTGCTAATACAAA AACA), primer B (AACCATCATGGAGAAAACTTCAGAG), primer C (CT CTGAAGTTTTCTCCATG), and primer D (AGCGGTAGCGACATCCT CCAT). Primers A and D are the outer primers. Primers B and C are the internal primers; these primers are complementary to each other and both contain the point mutation (underlined) that changes codon 131 of *RNR4* from TAC, encoding tyrosine, to TTC, encoding phenylalanine. Two separate PCRs with pRC2 as template DNA were performed with primers A and B and with primers C and D, respectively. The products of these reactions were mixed and subjected to another round of PCR with primers A and D. The *Bgl*II-*Eco*RI fragment of the final PCR product was used to replace the *Bgl*II-*Eco*RI fragment of pRC2, creating pWP155, a YCp plasmid containing the *rnr4-2* allele. DNA sequencing confirmed that the desired mutation had been created.

Immunofluorescence microscopy. Cells were prepared for immunofluorescence microscopy as described previously (23). Rabbit anti-β-tubulin polyclonal antibody 206 (1) was a gift from F. Solomon (Massachusetts Institute of Technology, Cambridge, Mass.). Fluorescein-conjugated goat antirabbit antibodies were obtained from Cappel Research Products (Durham, N.C.). Cellular DNA was visualized by staining with 4′,6′-diamidino-2-phenylindole (DAPI).

Flow cytometry. Diploid yeast cells were prepared for flow cytometry by the method of Hutter and Eipel (15). The *RNR4* diploid strain was made by mating CUY25 and CUY26. The *rnr4-Δ1* diploid strain was made by mating CUY995 and CUY996.

Measurement of induction of *RNR4*. Hydroxyurea or methyl methanesulfonate (MMS) was added to log-phase cultures of CUY26 cells at a final concentration of 0.1 M or 0.017%, respectively. Aliquots of 7.5×10^7 cells were collected at 0, 2, and 4 h after addition of drug. Cells were spheroplasted by treatment with 375 μg of Zymolyase (100T) per ml in 1.2 M sorbitol–0.1 M potassium phosphate (pH 7.5) at 30°C for 15 min. Spheroplasted cells were lysed by gentle vortexing in 0.5 ml of 4.3 M guanidinium thiocyanate. Total RNA was extracted by the one-step method (3). RNA was reconstituted in water treated with diethyl pyrocarbonate and quantified by spectrophotometry.

Three micrograms of RNA per lane was electrophoresed in a 1.4% formaldehyde agarose gel in morpholinepropanesulfonic acid buffer. RNA was then transferred to a GeneScreen membrane in 20× SSC (1× SSC is 15 mM sodium citrate plus 150 mM sodium chloride) and cross-linked to the membrane by baking at 80 to 100°C for 2 h. Hybridization probes for *RNR4* and *ACT1* were prepared by random priming with [α-^{32}P]dCTP. The blots were prehybridized for 4 h at 42°C in hybridization buffer containing 5× SSC, 5× Denhardt solution, 50% formamide, and 100 μg of single-stranded DNA per ml. Probes were added, and hybridization was carried out overnight. The blots were washed in 1.5× SSC–0.1% sodium dodecyl sulfate for 10 min at room temperature and then in 0.15× SSC–0.1% sodium dodecyl sulfate at 42°C for 20 min. mRNA levels were measured with a Fuji PhosphoImager. The amount of *RNR4* mRNA in each lane was normalized to the amount of *ACT1* mRNA in the same lane.

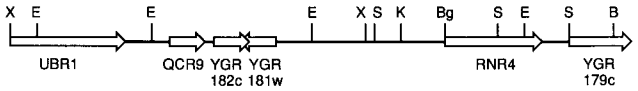

FIG. 1. Diagram of the pRC1 plasmid insert containing *RNR4*. Locations of restriction sites were determined by restriction mapping the pRC1 insert and subsequently confirmed by the complete sequencing of this region by the yeast genome project. The genomic sequence predicts an *Eco*RI site in YGR179c which we did not observe by enzyme digestion of our clone. The positions of open reading frames other than *RNR4* are also taken from the complete genomic sequence. *UBR1* and YGR179c extend beyond the ends of the insert. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Spe*I; X, *Xba*I.

Measurement of ribonucleotide reductase activity in yeast extracts. Yeast extracts from strains CUY26 and CUY995 were prepared as described by Vitols et al. (33) with the following modifications. Logarithmically growing yeast cells were cooled rapidly to 0°C, harvested, washed, and suspended in an equal volume of lysis buffer containing 0.05 M Tris-HCl (pH 7.5), 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 0.6 μM leupeptin, and 2 mM benzamidine. After disruption of the cells with a Bead-Beater (Biospec Products Inc., Bartlesville, Okla.), cell debris was removed by 10 min of centrifugation at 30,000 × *g*, and then the homogenate was centrifuged for 90 min at 320,000 × *g*. Solid ammonium sulfate (0.33 g/ml, nominally 55%) was added to the cleared supernatant to remove inhibitory deaminase and phosphatase activities (20). The precipitate was dissolved and equilibrated in the same buffer as described above, the ammonium sulfate was removed by chromatography on a Sephadex G-25 column, and the eluate was frozen in aliquots at −70°C. Protein concentrations were determined by the Bradford method (2). The ribonucleotide reductase assay mixture contained, in a total volume of 0.12 ml, 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM ATP, 10 mM dithiothreitol, 20 μM $FeCl_3$, 0.5 mM [5-^{3}H]cytidine diphosphate (specific activity, 37,000 cpm/nmol), 10 μM *E. coli* thioredoxin, and the fractionated cell extract (0.3 to 0.4 mg of protein). After incubation at 30°C for 30 min, the conversion of CDP to dCDP was determined as described earlier (9). Recombinant yeast Rnr1p and Rnr4p proteins were expressed in *E. coli* by using the pET expression system (31) and purified to homogeneity (2a).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper appears in the GenBank nucleotide sequence database under accession number U30385.

RESULTS

Identification of a second ribonucleotide reductase small subunit in yeast. Thomas et al. (32) isolated six yeast mutants that are cold sensitive for growth and resistant to levels of the microtubule-depolymerizing drug benomyl that inhibit the growth of wild-type cells. Complementation tests suggested that all of these strains contain a mutation in the same gene. One of these mutations, *tub2-104*, was shown to be single-base-pair change in the coding sequence of *TUB2*, which encodes β-tubulin, so all were referred to as *tub2* mutations. We have now shown that one of these mutations, originally called *tub2-158*, is not an allele of *TUB2*, and we have renamed it *rnr4-1* for reasons given below. In our hands, *rnr4-1* does complement the cold sensitivity of eight independent cold-sensitive alleles of *TUB2*, including *tub2-104*. In addition, we have shown that *rnr4-1* is not linked to the *TUB2* locus. The *rnr4-1* strain CUY58 was crossed to CUY848, which contains the *URA3* gene integrated adjacent to the *TUB2* locus. Following sporulation and tetrad dissection, the *URA3* marker segregated independently from cold sensitivity (parental ditype/tetratype/nonparental ditype ratio = 2:10:1). Similar crosses to CUY111 and CUY117 showed that *rnr4-1* is also unlinked to both the *TUB1* and *TUB3* genes, which encode the α-tubulins.

The wild-type *RNR4* gene was cloned by complementation of the cold-sensitive phenotype of *rnr4-1*. A yeast genomic library was transformed into CUY58, and transformants were selected for growth at 14°C. One plasmid, pRC1, which con-

TABLE 2. Amino acid identity between Rnr4p and R2 proteins of other organisms

Organism	% Identity[a]
Schizosaccharomyces pombe	52.1
Hamster	49.5
Mouse	48.6
Clam	48.2
Vaccinia virus	46.9
Urechis caupo	45.9
Dictyostelium	45.5
Human	45.4
Arabidosis	44.4
Caenorhabditis elegans	43.7
Plasmodium	41.8
E. coli	18.2

[a] Percentages were calculated by using the Megalign Program (DNASTAR, Inc.).

tains a ~7 kb insert (Fig. 1), was recovered. To determine the chromosomal origin of this insert, the *Bgl*II-*Bam*HI fragment from pRC1 was used to probe a lambda phage library containing clones of yeast genomic DNA (25). The probe hybridized to two overlapping clones (ATCC 70574 and 70647) which contain the *QCR9* gene on chromosome VII. Restriction mapping and partial DNA sequencing demonstrated that the *QCR9* gene is contained near one end of the insert in pRC1. The *rnr4-1* strain CUY58 was crossed to CUY977, which contains the *URA3* gene integrated at the *QCR9* locus. Sporulation and tetrad analysis indicated that *rnr4-1* and *QCR9* are tightly linked (parental ditype/tetratype/nonparental ditype ratio = 26:3:0). This result confirms that the *RNR4* gene, and not a dosage suppressor of *rnr4-1*, had been cloned.

The region of pRC1 that complements the cold sensitivity of *rnr4-1* was localized to a 2.4-kb *Bam*HI-*Kpn*I fragment at the end of the insert opposite *QCR9* (Fig. 1). Deletion of the ~850-bp internal *Spe*I fragment eliminates complementing activity, indicating that the *RNR4* gene is at least partially contained within this region. We sequenced ~1,500 bp of DNA from ~150 bp upstream of the *Bgl*II site to ~200 bp upstream of the second *Spe*I site. This region was found to contain a single 1,035-bp open reading frame that potentially encodes a 345-amino-acid, 40-kDa protein. Our sequencing was subsequently confirmed by the yeast genome sequencing project.

Rnr4p is similar to the small subunits of class I ribonucleotide reductases from a variety of organisms (Table 2). It is most similar to Rnr2p, the only other R2-like protein in *S. cerevisiae*. Rnr2p is a 399-amino-acid, 46-kDa protein that is essential for the viability of yeast (7, 14). It contains a 51-amino-acid N-terminal peptide that is lacking in Rnr4p. Aside from this sequence, the two proteins are 54% identical. The alignment is shown in Fig. 2 and requires three single-amino-acid gaps in the Rnr4p sequence.

The 16 amino acid residues that are conserved in all R2 proteins identified to date are indicated in Fig. 2. The radical-harboring tyrosine is at position 183 in Rnr2p. Rnr4p does possess a tyrosine residue in the equivalent position (amino acid 131) but contains substitutions at 6 of the remaining 15 conserved positions. Three of these substitutions affect amino acids that are directly involved in the coordination of the iron complex (17, 22). These include the two histidine residues in Rnr2p (at positions 179 and 276), which are both tyrosine residues in Rnr4p (at positions 127 and 223), and the glutamate residue in Rnr2p (at position 273), which is an arginine residue in Rnr4p (at position 230).

Cold-sensitive phenotype of *rnr4* mutants. To determine whether Rnr4p function is essential in yeast, we replaced the *RNR4* sequence with a DNA fragment containing the *HIS3* gene. The *RNR4* sequence replaced extended from the *Bgl*II site located ~50 nucleotides upstream of the start codon (the nearest open reading frame upstream of *RNR4* is ~2 kb away) to the *Eco*RI site located within the *RNR4* sequence encoding amino acid 302. This construct was transformed into the diploid strain CUY546. Two transformants were sporulated, and tetrads were dissected. Most tetrads contained four viable spores, indicating that the *RNR4* gene is not essential for the growth of yeast. However, cells containing the *RNR4* disruption (*rnr4-Δ1*) grow slowly; the generation time of *rnr4-Δ1* cells is about 50% greater than the generation time of *RNR4* cells.

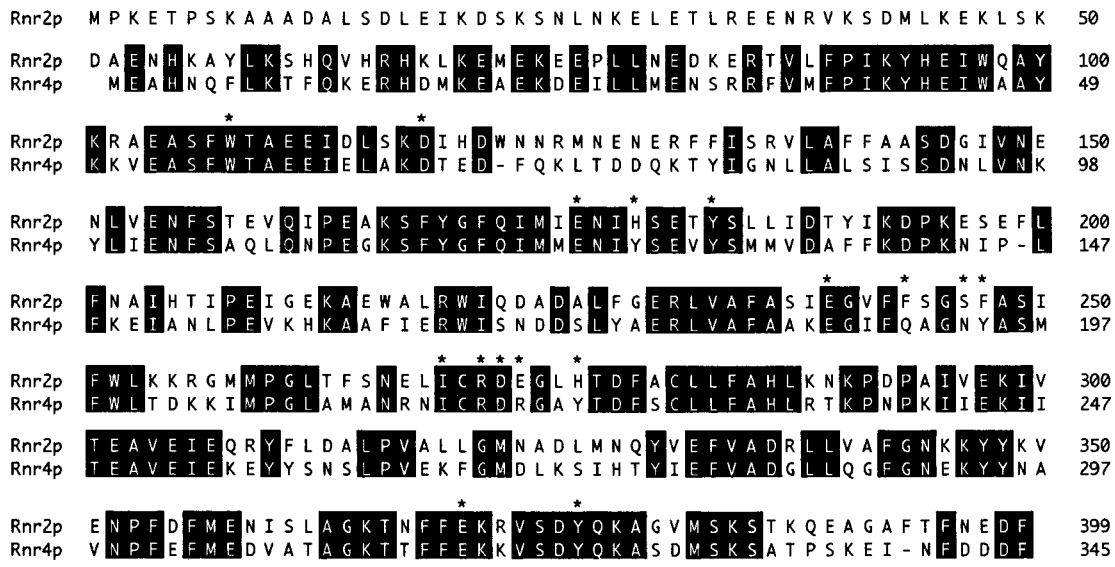

FIG. 2. Sequence alignment of Rnr4p and Rnr2p. Asterisks indicate the 16 amino acids that are conserved in all R2 proteins identified prior to Rnr4p. Rnr4p contains amino acid substitutions at six of these positions.

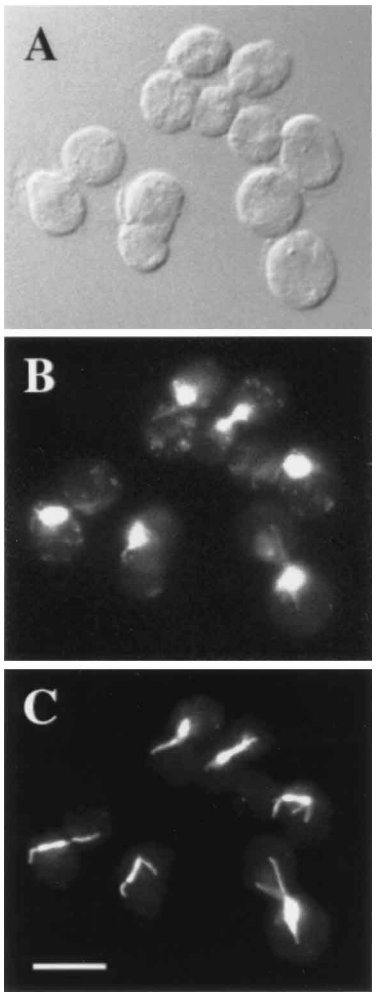

FIG. 3. Phenotype of *rnr4-1* cells at the restrictive temperature. CUY58 cells were grown at 30°C and shifted to 14°C for 16 h. (A) Nomarski optics; (B) DAPI staining of DNA; (C) indirect immunofluorescence with an antitubulin antibody. Images were photographed on T-Max 400 35-mm film, scanned into a Power Macintosh 7100 with a Polariod SprintScan 35 slide scanner, and compiled in Adobe Photoshop. Bar, 5 μm.

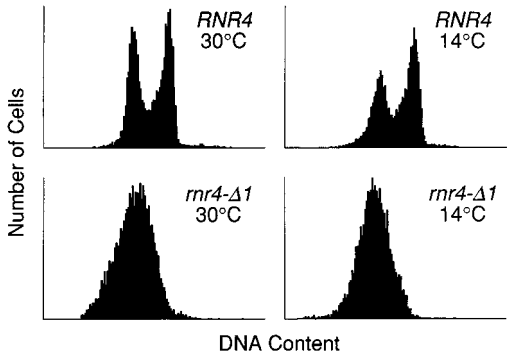

FIG. 4. Diploid cells homozygous for the indicated allele were grown at 30°C and shifted to 14°C for 16 h. The DNA content of individual cells was determined by flow cytometry.

Like the *rnr4-1* mutant, *rnr4-Δ1* cells are also cold sensitive and resistant to benomyl (see below).

Both *rnr4-1* and *rnr4-Δ1* cells fail to grow at 14°C and display a cell cycle arrest following a shift to this restrictive temperature. After 16 h at 14°C (about two generation times for *RNR4* cells at this temperature), greater than 80% of the cells in *rnr4-1* and *rnr4-Δ1* cultures were large budded. Most of these large-budded cells (80% for *rnr4-1* cells and 95% for *rnr4-Δ1* cells) contained a single undivided nucleus and a short preanaphase spindle (Fig. 3). Flow cytometry indicated that these cells are blocked prior to or in S phase (Fig. 4). Because the *rnr4-Δ1* mutation slows growth even at the permissive temperature, S-phase cells also accumulate at 30°C.

Drug sensitivity of the *rnr4* mutants. Ribonucleotide reductase is inhibited by hydroxyurea, which specifically quenches the enzyme's tyrosyl radical (19). We measured the sensitivity of cells to this drug by spotting suspensions of cells on plates containing various amounts of hydroxyurea. *RNR4* cells are able to grow on plates containing up to 100 mM hydroxyurea at 30°C. *rnr4-Δ1* cells are hypersensitive to hydroxyurea; they fail to grow on 25 mM hydroxyurea and grow only poorly on 10 mM hydroxyurea (Table 3). The *rnr4-1* mutant is also hypersensitive to hydroxyurea but less so than the deletion mutant; *rnr4-1* cells fail to grow on 100 mM and grow poorly on 50 mM hydroxyurea. Cells containing a heat-sensitive mutation in the *RNR2* gene (*rnr2-68*) are more sensitive to this drug than either of the *rnr4* mutants; the *rnr2-68* mutant fails to grow on 5 mM hydroxyurea, the lowest concentration tested. We could not test the drug sensitivity of cells lacking Rnr2p, because it is essential for growth.

The *rnr4-Δ1* cells are more resistant to benomyl than *RNR4* cells but not as resistant as *rnr4-1* cells. *rnr4-1* cells grow on plates containing 40 μg of benomyl per ml at 30°C, nearly twice the level that inhibits the growth of *RNR4* cells. *rnr4-Δ1* cells grow on 30 μg of benomyl per ml but not on 40 μg/ml. The *rnr2-68* mutation confers benomyl resistance similar to that produced by the *rnr4-Δ1* mutation. We found that this effect could be mimicked by treating wild-type yeast strains with hydroxyurea. In the presence of 25 or 50 mM hydroxyurea, CUY8 and CUY26 cells could grow on plates that also contained 40 μg of benomyl per ml. Thus, benomyl resistance appears to be a secondary consequence of inhibition of ribonucleotide reductase function. Perhaps limiting ribonucleotide reductase activity slows cell cycle progression during a phase that is critical for spindle formation, allowing cells more time to overcome the adverse effects of the drug on microtubule assembly.

Petite phenotype of *rnr4* mutants. The original *rnr4-1* mutant is a petite strain, a term used to describe yeast cells that lack mitochondrial function (11). In an attempt to recover *rnr4-1* grandes, we crossed the *rnr4-1* mutant to an *RNR4* grande strain, sporulated the resulting *RNR4*/*rnr4-1* diploid, and dissected tetrads. The petite phenotype (assayed as inability to grow on YPG plates) segregated 2:2, indicating a single nuclear gene defect, and cosegregated with cold sensitivity and benomyl resistance, indicating that the petite phenotype is due to the *rnr4-1* mutation. Thus, the *rnr4-1* cells become petite at a high rate. We observed the same result for *rnr4-Δ1* spores when *RNR4*/*rnr4-Δ1* diploids were sporulated and dissected.

We next tested whether the petite phenotype was a direct effect of the *rnr4-1* mutation or was a secondary effect of the mutation on the maintenance of a functional mitochondrial genome. The *rnr4-1* petite strain (CUY59) was crossed to an *RNR4* grande strain (DL2). This diploid is able to grow on YPG plates, indicating that the *rnr4-1* mutation is recessive for the petite phenotype. The *rnr4-1* petite strain (CUY59) was also crossed to an *RNR4 rho*^{0} strain (DL2rho^0) which lacks all mitochondrial DNA. The diploid product of this cross did not

TABLE 3. Temperature and hydroxyurea sensitivity of yeast strains

Relevant genotype		Relative growth rate[a] under the following conditions:									
		Temp (°C):					Hydroxyurea (mM):				
Chromosome	Plasmid	11	14	25	30	37	5	10	25	50	100
RNR4 RNR2	None	3	3	3	3	3	3	3	3	3	3
	RNR4	3	3	3	3	3	3	3	3	3	3
	RNR2	3	3	3	3	3	3	3	3	3	3
rnr4-1 RNR2	None	0	0	3	3	3	3	3	2	1	0
	RNR4	3	3	3	3	3	3	3	3	3	3
	RNR2	0	0	3	3	2	3	3	2	1	0
rnr4-Δ1 RNR2	None	0	0	2	2	1	3	1	0	0	0
	RNR4	3	3	3	3	3	3	3	3	3	3
	RNR2	0	0	2	2	0	1	0	0	0	0
RNR4 rnr2	None	3	3	3	2	0	0	0	0	0	0
	RNR4	1	2	3	2	0	0	0	0	0	0
	RNR2	2	2	3	3	3	3	3	3	3	3

[a] Relative growth rates: $3 > 2 > 1 > 0$.

grow on YPG plates, indicating that the *rnr4-1* haploid parent does not contain functional mitochondria. This result suggests that Rnr4p is required for proper maintenance of the mitochondrial genome.

Ribonucleotide reductase activity in *rnr4* mutant extracts. Wild-type yeast extract contains low levels of ribonucleotide reductase activity (Table 4). However, addition of bacterially expressed Rnr1p greatly stimulated this activity, indicating that the Rnr1p subunit is limiting in yeast extracts. Addition of bacterially expressed Rnr4p had little effect on the activity of wild-type extracts in either the absence or presence of added Rnr1p. Bacterially expressed Rnr1p and Rnr4p alone or in combination did not produce any detectable activity in the absence of yeast extract.

Ribonucleotide reductase activity was not detectable in *rnr4-Δ1* extracts. Addition of Rnr4p increased activity to the low level observed for wild-type extracts without addition of exogenous protein. With the addition of Rnr1p alone to the mutant extracts, ribonucleotide reductase levels increased but were four to five times lower than that of wild-type extracts with added Rnr1p. However, addition of Rnr4p and Rnr1p to the *rnr4-Δ1* extracts brought the ribonucleotide reductase levels up to that observed for wild-type extracts under the same conditions. Thus, the *rnr4-Δ1* mutant is deficient in ribonucleotide reductase activity, and this deficiency can be remedied by the addition of exogenous Rnr4p.

TABLE 4. Ribonucleotide reductase activity in yeast extracts

Relevant genotype	Yeast extract (μg)	Rnr1p (μg)	Rnr4p (μg)	dCDP formed (pmol)
RNR4	413	0	0	0.31
	335	30	0	4.3
	335	0	4.5	0.41
	335	30	1.4	5.0
rnr4-Δ1	413	0	0	ND[a]
	335	30	0	0.96
	335	0	4.5	0.32
	335	30	1.4	3.6

[a] ND, none detectable.

Induction of *RNR4* by hydroxyurea and MMS. Previous work has shown that the *RNR2* gene is induced by DNA-damaging agents (7, 13). We found that the DNA-damaging agent MMS strongly induces *RNR4* as well. RNA was prepared from yeast cells at 0, 1, and 2 h after exposure to MMS. Northern blot analysis showed that the *RNR4* transcript increased approximately 10-fold following treatment with MMS (Fig. 5). Treatment with hydroxyurea produced a similar level of *RNR4* induction (Fig. 5).

Rnr4p and Rnr2p are not functionally redundant. Because both *RNR2* and *RNR4* encode ribonucleotide reductase small subunits, we examined whether multiple copies of either one of these genes could compensate for mutations in the other gene. A wild-type copy of each gene on a high-copy-number 2μm YEp plasmid was transformed into *RNR4*, *rnr4-1*, *rnr4-Δ1*, and *rnr2-68* strains. These strains were scored for growth over a range of temperatures and on a range of concentrations of hydroxyurea (Table 3).

Neither gene on a high-copy-number plasmid has any effect on the temperature sensitivity or drug sensitivity of *RNR4* cells. The plasmid-borne *RNR4* gene complements the cold and hydroxyurea sensitivity conferred by both the *rnr4-1* and the

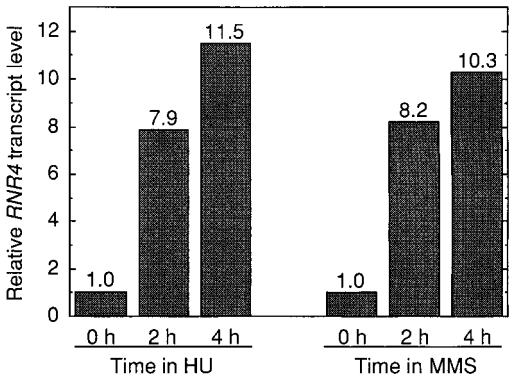

FIG. 5. Induction of the *RNR4* transcript by hydroxyurea and MMS. The graph shows the relative levels of *RNR4* mRNA before (0 h) and 2 and 4 h after exposure of cells to hydroxyurea (HU) or MMS. The amount of *RNR4* mRNA at 0 h was arbitrarily designated 1.0.

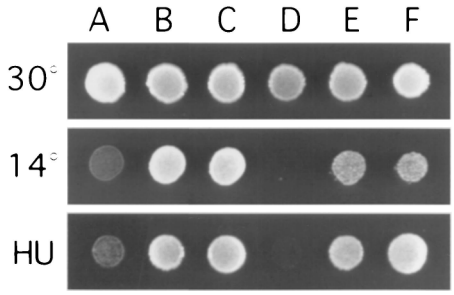

FIG. 6. Cells were spotted at low density onto SD plates lacking uracil and incubated at 30°C (top row) or 14°C (middle row) and onto a YPD plate containing 100 mM hydroxyurea (HU) and incubated at 30°C (bottom row). Lanes: A, *rnr4-1* cells containing a control YCp plasmid; B, *rnr4-1* cells containing *RNR4* on a YCp plasmid; C, *rnr4-1* cells containing *rnr4-2* on a YCp plasmid; D, *rnr4-Δ1* cells containing a control YCp plasmid; E, *rnr4-Δ1* cells containing *RNR4* on a YCp plasmid; F, *rnr4-Δ1* cells containing *rnr4-2* on a YCp plasmid. Images were captured on disks by using the FOTO/Analyst Archiver Electronic Documentation System (Fotodyne, Inc.), transferred to a Power Macintosh 7100, and compiled in Adobe Photoshop.

rnr4-Δ1 mutations. (*RNR4* on a YCp plasmid also complements these phenotypes.) Similarly, the plasmid-borne *RNR2* gene complements the heat and hydroxyurea sensitivity of the *rnr2-68* mutation. In contrast, the plasmid-borne *RNR2* gene does not complement the cold or hydroxyurea sensitivity caused by either *rnr4* mutation. In fact, high-copy *RNR2* increases the hydroxyurea sensitivity of the *rnr4-Δ1* strain and causes it to be slightly heat sensitive. Similarly, high-copy *RNR4* does not complement the heat or hydroxyurea sensitivity caused by the *rnr2-68* mutation but does make the *rnr2-68* strain somewhat cold sensitive.

Mutation of Tyr131 does not affect Rnr4p function. Although Rnr4p is quite similar to Rnr2p in overall sequence, it lacks a number of amino acid residues thought to be essential for coordination of the iron complex, which in turn is essential for generating the tyrosyl radical. This suggests that the cellular function of Rnr4p may not require the tyrosyl radical. To test this possibility, we changed the tyrosine at position 131 in Rnr4p to phenylalanine. Phenylalanine lacks the aromatic hydroxyl group of tyrosine and cannot form a radical. Otherwise, phenylalanine and tyrosine are identical, so this change will have a minimal effect on the structure of Rnr4p. The allele of *RNR4* containing the Tyr131Phe substitution is called *rnr4-2.*

The *RNR4* gene and the *rnr4-2* allele on yeast centromere-containing plasmids were transformed into *rnr4-1* and *rnr4-Δ1* yeast cells. Transformants were scored for their ability to grow in the cold and on 100 mM hydroxyurea. As shown in Fig. 6, the plasmid-borne copy of *rnr4-2* complements the cold sensitivity and hydroxyurea hypersensitivity of both the *rnr4-1* and *rnr4-Δ1* alleles. This result indicates that the cellular function of Rnr4p does not depend on its ability to form the tyrosyl radical.

DISCUSSION

We have identified a second *S. cerevisiae* protein with similarity to the small subunit of ribonucleotide reductase. Rnr4p is 54% identical to Rnr2p, 40 to 50% identical to R2 proteins from a number of other eukaryotic organisms, and distantly related to the *E. coli* R2 protein. Rnr4p does lack the N-terminal 51 amino acids of Rnr2p, but it is unlikely that this N terminus is needed for the enzymatic activity of Rnr2p. These N-terminal residues are absent from R2 proteins in many species, and truncation of N-terminal residues in mouse R2 does not significantly affect its enzymatic activity (21).

The phenotypes of the *rnr4* mutants are consistent with the idea that Rnr4p plays a role in ribonucleotide reductase function. *rnr4* mutants are hypersensitive to hydroxyurea, a drug that specifically inhibits ribonucleotide reductase. Yeast cells lacking *RNR4* grow slowly at optimal growth temperatures and fail to grow in the cold. At the restrictive temperature, *rnr4* mutants display an S-phase arrest similar to that observed following treatment of *RNR4* cells with hydroxyurea (8). *rnr4* mutants also lose mitochondrial function at a high rate, a phenotype previously observed for an *rnr2* mutant (7). A likely explanation for this latter phenotype is that the *rnr* mutations impede mitochondrial DNA replication by limiting the pools of deoxyribonucleotide precursors.

To determine directly whether Rnr4p plays a role in ribonucleotide reductase activity, we assayed this activity in extracts from wild-type cells and cells lacking Rnr4p. Wild-type extracts contain only low levels of activity, which are greatly stimulated by the addition of exogenous Rnr1p. The finding that Rnr1p is limiting in yeast extracts is consistent with the previous suggestion that the cell cycle regulation of ribonucleotide reductase activity is likely to be due primarily to fluctuation in the levels of *RNR1* message (8). The observation that *RNR4* deletion mutations can be suppressed by overexpression of *RNR1* also indicates that Rnr1p is limiting in vivo (12).

Cells lacking Rnr4p are deficient in ribonucleotide reductase activity. Extracts from *rnr4-Δ1* cells contain four- to fivefold-lower levels of this activity than wild-type cell extracts. The addition of exogenous Rnr4p to the mutant extracts increases their activity to wild-type levels. This result demonstrates that the reduction in ribonucleotide reductase levels in the mutant cells is a direct effect of the loss of Rnr4p.

The regulation of expression of *RNR4* is consistent with that expected for a gene encoding a subunit of ribonucleotide reductase. We found that the DNA-damaging agent and mutagen MMS strongly induces expression of the *RNR4* gene. *RNR4* thus shares with *RNR1*, *RNR2*, and *RNR3* the ability to be induced by agents that damage DNA (7, 8, 13).

The fact that Rnr2p and Rnr4p are similar in sequence suggests that they could be redundant in function. If this were the case, the phenotype associated with the loss of either gene could be due to an overall decrease in the levels of ribonucleotide reductase. The more severe phenotype associated with the loss of Rnr2p could be explained by hypothesizing that the *RNR2* gene produces more protein product than *RNR4*. However, neither gene on a high-copy-number plasmid can compensate for a mutation in the other gene. Thus, Rnr2p and Rnr4p do not perform equivalent functions in the cell. This result differs from that observed for the two yeast R1 proteins, Rnr1p and Rnr3p. The *RNR1* gene is essential for viability, but the *RNR3* gene is not. However, *RNR3* on a high-copy-number plasmid can suppress the lethality of *rnr1* mutations (8).

Although Rnr4p is clearly similar to ribonucleotide reductase small-subunit proteins from a variety of eukaryotic cells, it lacks a number of amino acid residues that are conserved in all other R2 proteins. Many of these residues are thought to be essential for coordination of the binuclear iron complex, which in turn is required for generation of the tyrosyl radical. The X-ray structure of *E. coli* R2 indicates that each iron atom is octahedrally coordinated by a histidine Nδ atom and five O atoms (22). These coordinating atoms are derived from six amino acid residues located at the iron-binding site and an O^{2-} ion. Five of these amino acids are conserved in all R2 proteins identified until now; the other is an aspartate residue in all R2 proteins except the Epstein-Barr virus protein, which

contains a glutamate residue at this site. Rnr4p has substitutions at three of these six positions. In particular, Rnr4p lacks the two histidine residues that bind to the iron atoms with their δ-nitrogen atoms. Both of these sites contain tyrosine residues in Rnr4p. The chemical and physical properties of tyrosine make it unlikely that this amino acid can substitute for histidine in the coordination of the iron complex. In addition, one of the glutamate residues that binds the iron complex via its carboxyl group is changed to an arginine residue in Rnr4p. Again, the obvious differences in the chemical properties of these two amino acids make it extremely unlikely that arginine could functionally replace glutamate in iron coordination. Thus, it would appear that these three amino acid substitutions preclude iron binding and, hence, tyrosyl radical formation by Rnr4p.

To determine more specifically whether tyrosyl radical formation plays a role in Rnr4p function, we changed the active-site tyrosine residue to phenylalanine. Phenylalanine lacks the aromatic hydroxyl group of tyrosine and therefore cannot form a radical. This tyrosine-to-phenylalanine mutation has no effect on the function of Rnr4p that we can observe. The mutant allele, *rnr4-2*, fully complements the cold sensitivity and hydroxyurea hypersensitivity of both the *rnr4-1* mutation and the *rnr4-Δ1* deletion.

The fact that the cellular function of Rnr4p does not require formation of a tyrosyl radical leads us to propose that this protein is enzymatically inactive. Nonetheless, the phenotypes of the *rnr4* mutants indicate that it does play an important role in ribonucleotide reductase function. One possibility is that Rnr4p acts as an antagonist to Rnr2p to modulate the level of reductase activity. In this role, Rnr4p could have a negative effect on reductase activity by forming enzymatically inactive complexes with Rnr1p or Rnr3p and effectively lowering the levels of these proteins in the cell. However, cells lacking Rnr4p have lower levels of ribonucleotide reductase activity, ruling out a negative role for Rnr4p. Alternatively, Rnr4p could have a positive effect on reductase activity by binding to and titrating out a negative regulator of Rnr2p. If Rnr4p functions in this manner, then one would predict that the relative levels of Rnr2p and Rnr4p would be critical. However, increasing the copy number of either *RNR2* or *RNR4* produced no observable phenotype in wild-type cells.

Therefore, we currently favor the hypothesis that Rnr4p is a nonenzymatic component of the active ribonucleotide reductase complex. We propose that the primary R2 complex in yeast is a heterodimer composed of Rnr2p and Rnr4p. Catalytic activity resides only in the Rnr2p subunit, but Rnr4p is an important structural component of the active tetramer. In support of this model, Huang and Elledge (12) have shown that Rnr2p and Rnr4p interact in vivo.

Such a situation is not unprecedented. In the bacterial photosynthetic reaction center, chromophoric groups are arranged with nearly perfect twofold symmetry (5). This symmetry arises because the L and M subunits, with which these groups are associated, have homologous sequences and similar structures. However, the two symmetry-related groups of chromophores are not functionally equivalent; electrons are almost exclusively transferred through the L subunit. This effect is attributed to subtle differences between the L and M subunits. A second pertinent example is reverse transcriptase, a dimeric protein of p66 and p51 (16, 18). Both subunits are initially synthesized as identical 66-kDa polypeptides. However, the RNase H domain of one of the two subunits is proteolytically excised, yielding a 51-kDa polypeptide. Although the polymerase domains of p66 and p51 are chemically identical, only the polymerase domain of p66 is active. Thus, reverse transcriptase is composed of two nearly identical polypeptides, but only one of these plays a catalytic role. Clearly, structural studies on the yeast ribonucleotide reductase will have to be performed before we can determine the relevance of these examples to Rnr2p and Rnr4p.

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