Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase

Stephen J. Elledge^{1,3} and Ronald W. Davis²

¹Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030 USA; ² Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305 USA

Ribonucleotide reductase activity is essential for progression through the cell cycle, catalyzing the rate-limiting step for the production of deoxyribonucleotides needed for DNA synthesis. The enzymatic activity of the enzyme fluctuates in the cell cycle with an activity maximum in S phase. We have identified and characterized two *Saccharomyces cerevisiae* genes encoding the regulatory subunit of ribonucleotide reductase, *RNR1* and *RNR3*. They share ~80% amino acid identity with each other and 60% with the mammalian homolog, M1. Genetic disruption reveals that the *RNR1* gene is essential for mitotic viability, whereas the *RNR3* gene is not essential. A high-copy-number clone of *RNR3* is able to suppress the lethality of *rnr1* mutations. Analysis of mRNA levels in cell-cycle-synchronized cultures reveals that the *RNR1* mRNA is tightly cell-cycle regulated, fluctuating 15- to 30-fold, and is coordinately regulated with the *POL1* mRNA, being expressed in the late G₁ and S phases of the cell cycle. Progression from the α -factor-induced G₁ block to induction of *RNR1* mRNA is blocked by cycloheximide, further defining the requirement for protein synthesis in the G₁- to S-phase transition. Both *RNR1* and *RNR3* transcripts are inducible by treatments that damage DNA, such as 4-nitroquinoline-1-oxide and methylmethanesulfonate, or block DNA replication, such as hydroxyurea. *RNR1* is inducible 3- to 5-fold, and *RNR3* is inducible >100-fold. When *MATa* cells are arrested in G₁ by α -factor, *RNR1* and *RNR3* mRNA is still inducible by DNA damage, indicating that the observed induction can occur outside of S phase. Inhibition of ribonucleotide reductase activity by hydroxyurea treatment results in arrest of the cell cycle in S phase as large budded, uninucleate cells. This specific cell-cycle arrest is independent of the *RAD9* gene, defining a separate pathway for the coordination of DNA synthesis and cell-cycle progression.

[Key Words: Ribonucleotide reductase; DNA-damaging agents; DNA synthesis; cell-cycle progression]

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The eukaryotic cell cycle is a cascade of highly complex, sequential, independent, and interdependent events that culminate in the duplication of a cell. Many complex macromolecular structures must be assembled and disassembled with striking temporal and spatial precision. This degree of complexity necessitates the existence of a sophisticated regulatory network that is capable not only of coordinating these events but also of correcting mistakes that occur during these complex processes. One level of this organization is accomplished through the restriction of certain cell-cycle functions to particular periods of the cell cycle when they are needed. For example, DNA synthesis is restricted to a defined period of the cell cycle, S phase (Pringle and Hartwell 1981). This restriction is accomplished, at least in part, by the temporal modulation of the activity and expression of gene products needed specifically in S phase. In *Saccha-*

romyces cerevisiae, not only are genes encoding the enzymatic machinery for DNA synthesis cell-cycle regulated [for example *POL1*, DNA polymerase I (Johnston et al. 1987); *CDC9*, DNA ligase (Barker et al. 1985; Peterson et al. 1985)], but so are many of the enzymatic activities involved in the production of the dNTP precursors needed for DNA synthesis [for example, *CDC8*, thymidylate kinase (White et al. 1988); *CDC21*, thymidylate synthase (Storms et al. 1984) and ribonucleotide reductase (Lowden and Vitols 1973)]. Several other genes associated with DNA metabolism are also cell-cycle regulated; these include histones (Hereford et al. 1981); *HO*, an endonuclease involved in mating type switching; *SW15*, a regulator of mating type switching (Nasmyth 1987); and *RAD6* (Kupiec and Simchen 1986). All of these genes are expressed in late G₁ and/or S phase. In each case, with the exception of *RAD6*, which has not been tested, these genes are not expressed when cells are arrested in early G₁ by a *cdc28* mutation or α -

³Corresponding author.

factor. *HO*, *CDC8*, *CDC9*, *CDC21*, and *POL1* are expressed immediately upon reaching the start of the cell cycle in late G₁, before the *CDC4* block. The *HTA1* and *HTB1* genes are expressed in S phase after the *CDC4* block. This regulation could occur at the level of mRNA synthesis or degradation, or both. In yeast, the only genes that have been examined so far, *HO* (Nasmyth 1985), *CDC21* (McIntosh et al. 1988), and *HTA1* and *HTB1* (Osley et al. 1986), are regulated at the level of mRNA synthesis, although post-transcriptional regulation is important for histone regulation in higher eukaryotic cells (Schumperli 1986). Positive- and negative-acting sequences are involved in cell-cycle regulation of the histone *HTA1* and *HTB1* genes (Osley et al. 1986), and mutations in the *HIR* genes have been identified that alter the negative regulation (Osley and Lycans 1987). *hir* mutations do not affect the regulation of the late G₁ class of genes. The *HO* gene encodes an endonuclease that is involved in mating type switching. It is under several other types of control in addition to cell-cycle regulation and has an extremely complicated regulatory region. In the *HO* promoter, a repeated sequence, CACGA₄, has been found to confer cell-cycle regulation on a heterologous promoter (Nasmyth 1985). This repeat has not been found in any other cell-cycle-regulated genes. A DNA-binding factor has been identified that binds this sequence in a *SW14*- and *SW16*-dependent fashion (Andrews and Herskowitz 1989), but if and how that factor is cell-cycle-regulated is not known. The sequences regulating the remaining genes of the late G₁ class have not been identified.

As an initial approach to the problem of cell-cycle regulation, we chose to examine that of 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, the small subunit is encoded by *RNR2* (Elledge and Davis 1987; Hurd et al. 1987). The activity of the enzyme fluctuates in the cell cycle with a maximum in S phase (Lowden and Vitols 1973). The amount of ribonucleotide reductase is also increased under conditions of nucleotide depletion (Lammers and Follman 1984). This increase in activity is likely due, in part, to increased transcription of the *RNR2* gene, which has been studied extensively (Elledge and Davis 1989a,b; Hurd and Roberts 1989). 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. The induction of *RNR2* mRNA can occur in G₁ or S phase and is not blocked by the presence of cycloheximide. DNA from the promoter region of *RNR2* can confer DNA damage inducibility upon a heterologous promoter. DNA damage inducibility is a feature common to several S-phase-specific genes (*POL1*, *CDC8*, and *CDC9*) and may be connected to their cell-cycle regulation. To understand fully the regulation of ribonucleotide reductase in the cell cycle, we isolated the genes encoding the regulatory (large) subunit of the enzyme. This paper describes the isolation of these genes, their regulation in the cell

cycle and by DNA-damaging agents, the phenotypes of mutations in these genes, and the effects of inhibition of ribonucleotide reductase on the cell cycle.

Results

Two genes, RNR1 and RNR3, encode alternate regulatory subunits of ribonucleotide reductase

Previous sequence analysis of the *RNR2* gene encoding the small subunit of ribonucleotide reductase revealed that the yeast gene shared 60% sequence identity with its mammalian homolog (Elledge and Davis 1987). This suggested that if the genes encoding the large regulatory subunits were equally related, then the yeast gene could be isolated by using the previously cloned mouse gene as a probe (Caras et al. 1985; Thelander and Berg 1986). A 1.5-kb *Bam*HI fragment from the mouse large-subunit gene was used to probe a genomic library of yeast DNA in λ at a reduced stringency, as described in Materials and methods. Two classes of cross-hybridizing λ clones were obtained that differed in restriction maps (Fig. 1). The stringency-dependent degree of hybridization appeared to be the same for both classes of positives, suggesting that they shared an approximately equal percentage sequence identity with the mouse gene (data not shown). Furthermore, they each showed cross hybridization with probes derived from different regions of the

Figure 1. Restriction maps of the *RNR1* and *RNR3* genes. The solid lines represent chromosomal DNA. The shaded boxes represent the coding regions of the genes. The arrows indicate the direction of transcription. Restriction endonuclease cleavage sites are noted above the appropriate positions in the genes. Restriction enzyme sites are abbreviated as follows: (Bam) *Bam*HI; (Bgl) *Bgl*II; (R1) *Eco*RI; (H3) *Hind*III; (Kpn) *Kpn*I; (Sac) *Sac*I; (Sal) *Sal*I; (Xho) *Xho*I. The *URA3* and *TRP1* genes shown above the *RNR* genes show the sizes and positions of insertions used to create the chromosomal disruptions *rnr1::URA3* and *rnr3::TRP1*. (A and B) Regions of the *RNR1* and *RNR3* genes that have been sequenced in both strands. This information was used to derive the deduced amino acid sequence shown in Fig. 2.

coding region of the mouse gene (data not shown), suggesting that the homology to the mouse gene was extensive.

Partial sequence analysis of the two genes revealed that they shared sequence identity throughout their lengths. The two yeast genes shared ~80% sequence identity with each other and ~60% identity to the mouse gene at both the nucleic acid and amino acid level (Fig. 2). The genes were named *RNR1* and *RNR3*, respectively. RNA hybridization analysis determined that the *RNR1* gene encoded a 3.2-kb mRNA, whereas no transcript was detectable for the *RNR3* gene (data not shown, see below).

The RNR1 gene is essential for mitotic viability and gives a CDC terminal phenotype upon disruption

To determine the phenotypes of mutations in *RNR1* and *RNR3*, disruptions of these genes were created in vitro by introduction of a selectable marker into the coding region of the two genes. These disruptions, *rnr1 :: URA3* and *rnr3 :: TRP1* (Fig. 1), were then introduced into the diploid yeast strain YNN402 by the method of transplacement (Rothstein 1983), selecting for Ura and Trp prototrophy, respectively. Disruptions were verified by Southern blot analysis. Two independently derived dip-

loids heterozygous for respective *rnr* mutations were allowed to undergo sporulation, and tetrad analysis was performed (Table 1). Of the 27 tetrads analyzed for the *rnr1 :: URA3* disruption, no tetrads with three or four viable spores were observed (Table 1). Twenty-five tetrads had two viable spores, and two tetrads had one viable spore, and all viable spores were Ura auxotrophs. Microscopic examination of the inviable spores revealed that they did germinate and undergo one or two rounds of cell division before arresting as large budded cells, a classical CDC phenotype associated with mutations in genes involved in DNA synthesis.

Of the 32 tetrads analyzed for the *rnr3 :: TRP1* disruption, 29 had four viable spores and 3 had three viable spores (Table 1). The *TRP1* gene segregated 2 : 2, indicating that the *RNR3* gene is not essential for mitotic viability. The *rnr3 :: TRP1* haploids did not appear to have a growth defect relative to their *RNR*⁺ sister spore clones.

High-copy numbers of the RNR3 gene can suppress mutations in the RNR1 gene

Although *RNR1* and *RNR3* share considerable sequence homology, *rnr3* mutants had no observable phenotype and *RNR3* message was not detectable in vegetatively growing cells. This suggested that the *RNR3* gene may not encode a functional large subunit of ribonucleotide reductase. To search further for a function for *RNR3*, the *RNR3* gene was placed on the *TRP1*-containing 2- μ m circle vector pRS46 to create pNN449 and tested to determine whether it could suppress a null mutation in *RNR1*. To facilitate this analysis, the sectoring shuffle assay developed by Elledge and Davis (1988) was employed. The *RNR1* and *SUP11* genes were placed on a centromeric vector, pUN95 (Elledge and Davis 1988), containing the *HIS3* gene to create pNN448 and introduced into a haploid yeast strain YNN405 bearing an *ade2* ochre mutation, producing YNN406. *ade2* mutations cause the accumulation of a red pigment in strains grown under limiting adenine conditions. The presence of the *SUP11* gene suppresses the *ade2* mutation and prevents the formation of the red color. YNN406 colonies appear white when grown on limiting adenine medium lacking histidine but have red sectors when grown in the presence of histidine due to growth of cells that have lost the plasmid. Replacement of the chromosomal *RNR1* gene in YNN406 with the *rnr1 :: URA3* mutation resulted in homogeneously white colonies when grown on limiting adenine medium supplemented with histidine and uracil, indicating that loss of the plasmid *RNR1* gene is lethal, as predicted by the tetrad analysis. Introduction of pNN449, which contains the *RNR3* gene, into this strain restored the ability of the strain to grow in the absence of pNN448 and produced sectoring colonies. Southern analysis confirmed the genetic interpretation (Fig. 3); presence of a high-copy number of the *RNR3* gene can suppress null mutations in *RNR1*, demonstrating that the *RNR3* gene encodes a functional regulatory subunit of ribonucleotide reductase.

Figure 2. Comparison of the deduced amino acid sequence of portions of the mouse (M1) and yeast (*RNR1* and *RNR3*) genes encoding the large subunit of ribonucleotide reductase. Dashes represent amino acid identities to the amino acid sequence of the *RNR1* gene. Dots represent sequences that have not been determined. (A) The deduced amino acid sequence of the amino-terminal region of the large subunits, including the start of translation, corresponding to the region labeled A in Fig. 1. (B) The deduced amino acid sequence of an internal region of the large subunits corresponding to the region labeled B in Fig. 1.

Table 1. Tetrad analysis of *RNR1* and *RNR3* mutants

Strain	Number of viable spores per tetrad	Number of tetrads observed	Genotype			
			<i>ura3</i>	<i>URA3</i>	<i>trp1</i>	<i>TRP1</i>
YNN403	4	0	0	0		
	3	0	0	0		
	2	25	50	0		
	1	2	2	0		
YNN404	4	29			58	58
	3	3			5	4
	2	0			0	0
	1	0			0	0

RNR1 and *RNR3* are both inducible by DNA damage

Transcription of the *RNR2* gene is inducible by a variety of treatments that damage DNA or block DNA synthesis. In addition, a number of other genes whose products are involved in DNA synthesis, such as *CDC8* (Elledge and Davis 1987), *CDC9* (Barker et al. 1985), and *POL1* (Johnston et al. 1987), are induced by DNA-damaging treatments. To examine whether the *RNR1* and *RNR3* genes share this mode of regulation, RNA was prepared from logarithmically growing cells with and without treatment with the DNA damaging agent 4-nitroquinoline-1-oxide (4-NQO). RNA hybridization analysis performed under stringent hybridization conditions showed that transcripts for both *RNR1* and *RNR3* were inducible by 4-NQO treatment (Fig. 4). Induction of the *RNR1* transcript was ~3- to 5-fold, whereas the *RNR3* transcript was inducible >100-fold. Analysis of densitometric tracings from greatly overexposed films has not allowed detection of a message from uninduced cells, suggesting that transcription of *RNR3* is completely repressed in the absence of inducing treatments. The fact that *RNR3* is not normally expressed provides a simple explanation of why *RNR3* mutants appear to have no growth defects.

Figure 3. Demonstration of suppression of the *rnr1 :: URA3* mutation by high-copy number of the *RNR3* gene through the use of the sectoring shuffle. Shown is a Southern blot analysis of DNA prepared from strains used in the sectoring shuffle. DNAs were digested with *EcoRI*, electrophoresed through a 1% agarose gel, transferred to nitrocellulose, and probed with the *EcoRI*-*BglIII* fragment of the *RNR1* gene containing the start of translation. The sources of DNA are as follows: (Lane 1) YNN405; (lane 2) YNN407; (lane 3) YNN409.

Transcription of RNR1 is tightly cell-cycle regulated

The enzymatic activity of ribonucleotide reductase fluctuates in the cell cycle with an activity maximum in S phase in all eukaryotic cells that have been examined, including yeast (Lowden and Vitols 1973). In mammalian cells, this regulation is accomplished by modulating the levels of the small subunit, whereas the large subunit remains constant throughout the cell cycle (Engstrom et al. 1985). To determine whether modulation of transcript levels is involved in the cell-cycle regulation of ribonucleotide reductase activity in yeast, RNA was prepared from a yeast culture undergoing synchronous progression through the cell cycle. The yeast strain LN114 was arrested in G₁ by treatment with α -factor. After 90–95% of the cells were arrested, the α -factor was removed, and the cells proceeded synchronously through the cell cycle. Samples of cells were taken at 10-min intervals, and RNA was prepared and subjected to RNA blot analysis, with the *RNR1*, *RNR2*, *POL1*, and *URA3* genes as probes (Fig. 5). The *POL1* gene is cell-cycle regulated and serves as an internal clock for timing the cell cycle and as a control for synchronization. The *URA3* gene is not cell-cycle regulated and serves as a control for the amount of RNA loaded in each lane. The *RNR1* transcript is tightly cell-cycle regulated over several cycles and is coordinately expressed with the *POL1* gene (Fig. 5), having an abundance maximum in S phase. The *RNR2* transcript shows a weak, twofold fluctuation of levels relative to the *URA3* transcript when analyzed by densitometer scanning of this and other RNA blots (data not shown). The fact that *RNR1* message increases significantly in the first time point indicates that the factors controlling its abundance must sense a very early event after cells pass "start". These data suggest that the cell-cycle regulation of ribonucleotide reductase activity is likely to be due primarily to fluctuation in the levels of *RNR1* message.

Progression from G₁ to RNR1 message induction requires protein synthesis

The pioneering work of Hereford and Hartwell demonstrated that progression of the cell cycle from G₁ to S phase requires protein synthesis (Hereford and Hartwell 1974). The fact that mRNAs for several genes required

for DNA synthesis, such as *POL1*, *CDC8*, *CDC9*, *CDC21*, and *RNR1*, are cell-cycle regulated provided a potential explanation for this observation, namely that these newly synthesized transcripts must be translated for DNA synthesis to be detected. Another possible explanation is that progression from G_1 to the induction of these transcripts, themselves, is dependent on protein synthesis. To test this hypothesis, cells were arrested in G_1 with α -factor, incubated with cycloheximide, and then released from the α -factor block while maintaining the presence of cycloheximide. RNA was prepared from these cells at various times after removal of the α -factor, and RNA hybridization analysis was performed (Fig. 6). The control cells were not treated with cycloheximide and showed the anticipated fluctuation of *RNR1* levels. However, the presence of cycloheximide completely prevented the accumulation of *RNR1* transcript. Similar results were obtained for the *POL1* message (data not shown). These data demonstrate that the protein synthesis block to entry into S phase occurs before induction of cell-cycle-regulated transcription or transcript accumulation.

Induction of *RNR1* and *RNR3* transcription by DNA-damaging agents is independent of the cell cycle

One explanation why ribonucleotide reductase and other genes whose activities are cell-cycle regulated respond to DNA damage is that they are needed outside of S phase to produce a metabolic state that facilitates DNA synthesis needed for repair processes. That is, the DNA-damage sensory network provides a mechanism for heterochronic expression of these genes. This hypothesis would predict that *RNR* induction occurs throughout the cell cycle. To test this hypothesis, the yeast strain RC634 was arrested in the G_1 phase of the cell cycle with the peptide hormone α -factor (Buckingham et al. 1973). Arrested cells were then treated with the carcinogen methylmethanesulfonate (MMS), a methylating agent, and hydroxyurea (HU), a specific inhibitor of ribonucleotide reductase, as described in Materials and methods, while maintaining the presence of α -factor. A population of asynchronous cells was used as a control. RNA was extracted from these samples after 4 hr of treatment with the inducing agents and size-fractionated, and the amount homologous to the *RNR1*, *RNR3*, and *URA3* genes was determined (Fig. 7). The message levels for both genes appear to increase relative to *URA3* message levels in response to each inducing agent in both cycling and G_1 -arrested cells. Densitometer tracing of lighter exposures of *URA3* reveals that lane 1 was underloaded by a factor of 2.3 relative to lane 4. The *RNR1* probe had a lower specific activity than the *RNR3* probe used in this experiment. Interestingly, induction levels for *RNR3* message by HU and MMS were significantly lower in α -factor-arrested cells than in the cycling population, whereas induction levels for *RNR1* appeared slightly higher in α -factor-arrested cells.

If HU generates a damage signal by stalling DNA replication through a depletion of dNTPs, it would be sur-

Figure 4. Northern blot analysis of the induction of *RNR1* and *RNR3* messages by 4-NQO. Individual cultures of *S. cerevisiae* SK46 were grown to an OD_{600} of 1.0, and 4-NQO was added to a final concentration of 0.25 μ g/ml. Cultures were incubated with shaking at 30°C for 0, 30, and 60 min, after which cells were harvested and RNA was prepared. The numbers above each lane indicate the length of time each sample was exposed to 4-NQO. The gene name (top) represents the probe used. Probes are described in Materials and methods.

prising to see induction in the absence of S-phase-dependent DNA replication. However, mitochondrial DNA replication proceeds in a cell-cycle-independent manner and may be responsible for the generation of a stress signal if nucleotide levels are not maintained in the presence of HU. It is also possible that nucleotide pools turn over at a significant rate in the absence of DNA replication and that nucleotide levels may play a role in the signaling. The fact that induction by HU was lower in G_1 -arrested cells for *RNR3* suggests that an S-phase-dependent function, perhaps chromosomal DNA replication, may contribute to the stress signal produced by HU, or perhaps the *RNR3* transcripts are less stable in G_1 than in the rest of the cell cycle. Clearly, these data show that DNA damage induction can occur outside of S phase, supporting the heterochronicity hypothesis.

Blocking DNA replication arrests the cell cycle through a *RAD9*-independent pathway

Organisms show a specific arrest of the cell cycle at the G_2 -to-M transition in response to DNA damage (Burns 1956). In *S. cerevisiae*, this response pathway is controlled by the *RAD9* gene. *rad9* mutants are sensitive to killing by a number of DNA-damaging agents and fail to show the specific cell-cycle arrest in response to DNA

Figure 5. *RNR1*, *RNR2*, and *POL1* levels in a culture of cells synchronized by α -factor treatment. LN114 cells were grown to OD₆₀₀ of 0.5 and synchronized in G₁ by α -factor treatment, as described in Materials and methods. After cells accumulated in G₁, α -factor was removed and cells progressed synchronously through the cell cycle. Cells were harvested at 10-min intervals and RNA was prepared, fractionated on a formaldehyde/1% agarose gel, transferred to nitrocellulose, and probed. The numbers (top) represent the time after release from α -factor. (Left) The gene name represents the probe used. Probes are described in Materials and methods.

damage (Weinert and Hartwell 1988). *rad9* mutants have an "anti-*CDC*" phenotype with respect to several *CDC* genes such as *cdc2* and *cdc9* (Weinert and Hartwell 1989). *RNR1* and *RNR2* mutations arrest with the same terminal phenotype as *cdc2* mutant, singly budded, uninucleate cells. The specific inhibitor of ribonucleotide reductase, HU, also arrests cells with the same *CDC* phenotype (Slater 1973). Because HU treatment appears to be capable of activating the same stress response pathways that many DNA-damaging agents activate, it seemed possible that it may achieve its cell-cycle arrest via the *RAD9*-dependent pathway. To test this hypothesis, isogenic *Rad*⁺ and *rad9* cells were treated with HU, and the cell-cycle stage distribution of the cells was determined (Table 2). The presence of the *rad9* mutation

had no effect on the cell-cycle arrest induced by HU treatment. These data demonstrate that inhibition of DNA synthesis causes a cell-cycle arrest that is independent of the *RAD9* gene and thus further defines the cellular checkpoint responsible for coordinating DNA synthesis and mitosis. These data do not rule out the possibility that the HU treatment may be capable of activating the *RAD9*-dependent cell-cycle-arrest pathway.

Discussion

Two genes encode the regulatory subunit of ribonucleotide reductase in S. cerevisiae

The regulatory subunit of ribonucleotide reductase has two sites for allosteric regulation of the enzymatic ac-

Figure 6. Progression from the G₁-induced α -factor block to expression of *RNR1* is dependent on protein synthesis. LN114 cells were grown to OD₆₀₀ of 0.5 and synchronized in G₁ by α -factor treatment, as described in Materials and methods. The culture was split, and cycloheximide was added to one sample to a final concentration of 100 μ g/ml. Cells were incubated with the drug for 10 min, and the α -factor was removed while maintaining the presence of cycloheximide. The other sample was handled identically but was never treated with cycloheximide. Cells were harvested at 0-, 20-, 50-, and 80-min intervals, and RNA was prepared, fractionated on a formaldehyde/1% agarose gel, transferred to nitrocellulose, and probed. The numbers (top) represent the time in minutes after release from α -factor. (C) RNA from cycling cells that were not treated with α -factor. (Right) With the exception of C, lanes are from cells treated with cycloheximide. Treatment of cycling cells with cycloheximide alone does not reduce the *RNR1* message (data not shown). The gene

name at left of each band represents the gene encoding that transcript. Probes are described in Materials and methods. A higher degree of synchrony was obtained in this experiment than in the experiment shown in Fig. 5.

Figure 7. Induction of the *RNR1* and *RNR3* mRNA by HU and MMS in growing cells and during G_1 arrest by α -factor. RNA was extracted from untreated RC634 cells (lane 1), 100 mM HU-treated cells (lane 2), 0.01% MMS-treated cells (lane 3), and from α -factor-arrested RC634 cells with no damage treatment (lane 4), arrested cells treated with 100 mM HU (lane 5), and arrested cells treated with 0.01% MMS (lane 6). Northern blots of these RNAs were hybridized with 32 P-labeled *RNR1*-, *RNR3*-, and *URA3*-specific probes, as described in Materials and methods, and the transcripts were visualized by autoradiography. Lane 1 was underloaded by a factor of 2.3 relative to lane 4.

tivity. One site is responsible for balancing the nucleotide pools by altering the substrate specificity of the enzyme in response to nucleotide concentrations, and the second allosteric site is involved in feedback regulation, measuring the dATP/ATP ratio and modulating the overall activity of the enzyme. Two genes, *RNR1*, and *RNR3*, encode the regulatory subunit of ribonucleotide reductase in *S. cerevisiae*. Under normal vegetative conditions, only the *RNR1* gene is expressed, and thus the ribonucleotide reductase in the cell is of a homogeneous form, $\alpha_1\beta_2$. However, in the presence of DNA damage, the second large subunit gene is induced, producing at least one $\alpha_2\beta_2$ and possibly two $\alpha_1\alpha_2\beta_2$ additional forms of ribonucleotide reductase differing in their subunit composition. The physiological role of *RNR3* remains to be determined. The yeast strains mutant for *RNR3* have no obvious growth defects and are not sensitive to HU or DNA-damaging agents (S.J. Elledge and R.W. Davis, unpubl.). Furthermore, the yeast strain that lacks *RNR1* and overproduces *RNR3* is not sensitive to HU (S.J. Elledge and R.W. Davis, unpubl.), suggesting that the *RNR3* protein can substitute efficiently for the *RNR1* protein when overproduced. The term "overproduced" is used loosely here because expression levels of the protein have not been measured. What, then, is the role of *RNR3*? Clearly, it must confer some selective advantage for the cell to have conserved both its function and tight regulation. One possibility is that it has altered regulatory properties, allowing the cell to survive certain types of stress that have yet to be duplicated in the labo-

ratory. For example, biological fungicides are rampant in the wild and are often targeted to inhibit key regulatory enzymes. Perhaps, the evolutionary history of *S. cerevisiae* included growth in an ecosystem in which inhibitors of ribonucleotide reductase were a commonly employed competitive strategy. Duplication of the target gene would facilitate the rapid evolution of drug-resistant variants. Alternatively, the *RNR3* gene may play a role in a nonvegetative function of yeast such as meiosis. The true role remains to be determined.

Ribonucleotide reductase is cell-cycle regulated at the level of mRNA accumulation

Both the *RNR1* and *RNR2* transcripts appear to fluctuate through the cell cycle. The *RNR1* message is tightly cell-cycle regulated, with levels fluctuating 15- to 30-fold in experiments achieving a high degree of synchrony, whereas the *RNR2* message showed a modest 2-fold fluctuation. The *RNR1* message is reduced to very low levels in G_1 in contrast to the *RNR2* transcript. This difference may be a reflection of differing message stabilities for the two genes; *RNR1* mRNA must have a very short half-life to undergo this striking modulation of levels. Likewise, to observe fluctuation of enzymatic activity, at least one of the subunits of ribonucleotide reductase must have a short half-life. The regulation of the yeast genes is in contrast to the cell-cycle regulation of the mammalian enzyme in which transcription of the small subunit (M2) is modulated, whereas the transcript for the large subunit (M1) remains relatively constant. However, M1 is transcriptionally regulated, being repressed upon entry into G_0 during serum starvation (Mann et al. 1988). It is not clear whether the apparent cell-cycle regulation of *RNR1* is mechanistically related to the serum starvation response of mammalian cells. Analysis of how other eukaryotes differentially regulate this gene pair will provide an interesting study in the evolution of eukaryotic gene regulation.

This transcriptional regulation is likely to account for the cell-cycle regulation of the enzymatic activity but does not rule out contributing post-transcriptional mod-

Table 2. Cell cycle arrest of *S. cerevisiae* by HU is independent of RAD9

Cell-cycle stage	YNN410 (RAD ⁺) cells (%)		YNN411 (rad9::URA3) cells (%)	
	-HU	+HU	-HU	+HU
Unbudded	22	2	24	1
Small buds	27	1	28	1
Large buds	51	97	48	98

Cell cultures grown to mid-log phase in YPD were split, and half of each culture was made 200 mM HU. Cultures were then incubated for 4 hr at 30°C with shaking, at which time cultures were assayed for their distribution in the cell cycle by light microscopy. Small budded cells were defined such that the daughter bud was <25% of the size of the parent cell.

ifications. T lymphocytes are arrested in the G₁ phase of the cell cycle by exposure to cAMP. Albert and Nodzenski (1989) have shown that mammalian small subunit M2 is phosphorylated by a cAMP-dependent protein kinase and that this phosphorylation decreases the activity of the enzyme. They suggest that this modification may play a role in the cell-cycle arrest caused by cAMP. It is not yet known whether the yeast proteins are also kinase substrates or whether their stabilities are modulated in the cell cycle.

The *RNR1* transcript is coordinately regulated with that of the *POL1* gene within the resolution of these experiments, being induced in late G₁ directly after cells pass "start". This coordinate expression is appropriate because both functions are needed at the same time to promote DNA synthesis. This message accumulation could be due to modulation of the rate of transcription or modulation of message stability. Both mechanisms function to promote cell-cycle message accumulation in mammals. Although these genes are similarly regulated, it is not clear whether they share precisely the same regulation. Further experiments are needed to answer this question. Nevertheless, the early induction of these genes suggests that the *cis*-acting sequences responsible for their regulation must be sensing a very early event or events in the commitment to enter the cell cycle and will serve as tools to probe these early events.

Progression from the α -factor block to induction of RNR1 requires protein synthesis

The ability to induce the *RNR1* and *POL1* messages after arrest in G₁ is dependent on protein synthesis. This may explain the early observations of Hereford and Hartwell that progression from the α -factor-induced G₁ arrest to S phase required protein synthesis. Although further analysis is required to determine whether this is a general requirement of all G₁-arrested states or is specific to the α -factor arrest, it does raise some interesting possibilities. One possibility is that a new message transcribed immediately upon passing "start" must be translated to activate transcription of these genes. If this is true, a differential cDNA screen looking for transcripts expressed after the α -factor block but before the cycloheximide block should identify the gene of interest. Another interesting possibility is that a critical level of a protein product must accumulate for progression into S and activation of transcription and that the mechanism by which α -factor arrests the cell cycle is by specifically depleting or altering this protein. Cycloheximide would then act to block the accumulation of that protein after release from the α -factor block. The best candidate for such a protein is cyclin. Cyclins were identified because of their unusual behavior during the cell cycle (Evans et al. 1983). Cyclins accumulate during the cell cycle, peaking in abundance at the end of G₂. After cells enter mitosis, the cyclins are rapidly destroyed. Cyclins are thought to be involved in the activation of the kinase activity of p34, a protein kinase highly homologous to the *CDC28* protein of *S. cerevisiae*. *CDC28* regulates entry into S phase in *S. cerevi-*

siae, and three cyclin homologs have been identified that appear to be involved in this regulatory step: *WHI1*, also known as *DAF1* (Cross 1988; Nash et al. 1988), *CLN1*, and *CLN2* (Hadwiger et al. 1989). *WHI1* has been implicated in the α -factor-arrest pathway (Cross 1988; Courchesne et al. 1989). Depletion or physical alteration of cyclins could occur during α -factor arrest, thereby blocking the activation of *CDC28* causing cell-cycle arrest. Recovery would then be mediated by the cycloheximide-sensitive synthesis of new, unaltered cyclins.

RNR1 and RNR3 transcripts are inducible by agents that damage DNA or block DNA replication

Although many cell-cycle-regulated activities are required primarily in one stage of the cell cycle, circumstances can arise when these activities are needed outside of the period when they are normally expressed. Consequently, multiple types of regulation are often interwoven into the control of cell-cycle-regulated genes. The *RNR1* and *RNR3* genes join a large group of genes with cell-cycle-regulated activities involved in DNA synthesis that are inducible by agents that damage DNA or block DNA replication. 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 (Elledge and Davis 1987; Elledge and Davis 1989a). The fact that the common function shared by these genes is their role in DNA synthesis suggests that the cellular response regulating their expression is designed to promote efficient DNA synthesis. The ability of cells to synthesize DNA is high during S phase, and it is likely that the additional induced levels of these enzymes produced by DNA damage would not significantly alter the repair capacity of the cell in that stage. However, outside of S phase, the levels of these activities drop, and with them the capacity to synthesize DNA. The ability of this DNA damage sensory pathway to induce heterochronically the capacity to synthesize DNA outside of S phase is likely to be the major selective advantage to the cell. The overall objective of the cell-cycle regulation and the DNA-damage regulation is the same, to provide the capacity to synthesize DNA when it is needed. Therefore, these different regulatory pathways may share common components. Perhaps, the DNA damage sensory network activates gene expression via the existing cell-cycle regulatory circuit. This important possibility remains to be explored.

Ruby and Szostack (1985) identified a number of genes, *DIN* (damage inducible), whose levels of expression increase in response to DNA-damage treatments. One such gene, *DIN1*, was recently sequenced and found to be identical to *RNR3* (K. Yagle and K. McEntee, pers. comm.).

Cell-cycle arrest by inhibition of ribonucleotide reductase defines a RAD9-independent checkpoint for regulation of the cell cycle

Among the responses to the stress of DNA damage

shared among organisms is a specific arrest of the cell cycle at the G₂-to-M transition (Burns 1956). This response is thought to allow cells additional time to process and repair DNA damage prior to chromosome segregation, thus avoiding potential mitotic catastrophes associated with attempting to process incompletely repaired and replicated chromosomes. In *S. cerevisiae*, this response pathway is controlled by the *RAD9* gene. *rad9* mutants are sensitive to killing by a number of DNA-damaging agents and fail to show the specific cell-cycle arrest in response to DNA damage (Weinert and Hartwell 1988). Furthermore, *rad9* mutants have an anti-*CDC* phenotype with respect to several *CDC* genes such as *cdc2* and *cdc9* (Weinert and Hartwell 1989). *RNR1* and *RNR2* mutations, as well as the specific inhibitor of ribonucleotide reductase HU, arrest cells with the same terminal phenotype as *cdc2* mutants, singly budded, uninucleate cells. HU-induced cell-cycle arrest, however, is not dependent on the *RAD9* gene. This result formally differentiates these two cell-cycle arrest pathways for yeast. Although the *RAD9* gene appears to coordinate some aspects of DNA metabolism and cell-cycle progression, it cannot be responsible for the primary sensing of DNA replication progression because *rad9* null mutations have no striking mitotic phenotypes in the absence of DNA damage. The HU-induced arrest pathway is more likely to be that involved in the coordination of DNA synthesis and mitosis. If this is true, we would predict that any agent arresting the yeast cell cycle with *CDC* phenotype by blocking DNA replication would also arrest in a *RAD9*-independent manner, via this S phase coordination checkpoint. In support of this, Schiestl et al. (1989) have found that *CDC8* retains its *CDC* phenotype in a *rad9* background.

Ribonucleotide reductase plays a central role in the eukaryotic cell, being responsible for both producing and balancing the deoxynucleotide pools needed for DNA synthesis. Mutations in it have been linked to a variety of phenotypes, including increased spontaneous mutation rates, loss of mitochondrial function, and DNA repair deficiencies. Ribonucleotide reductase is regulated by the cell cycle and is, itself, an indirect regulator of the cell cycle; progression from G₁ to S is needed to activate ribonucleotide reductase expression, and active ribonucleotide reductase is needed to progress from S to G₂. In addition to sensing cell-cycle positional information, it is capable of sensing the structural integrity of the DNA itself, being induced in response to DNA damage or blocks in DNA replication. The central themes of this work, cell-cycle progression, cell-cycle-regulated gene expression, and DNA-damage responsive gene expression, can all be approached through the study of the *RNR* genes encoding ribonucleotide reductase.

Materials and methods

Media and chemicals

Yeast minimal medium contains 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI); 2% glucose and 2% Bacto-Agar (Difco) are added for solid media. Se-

lective medium used is minimal medium supplemented with various amino acids and bases, as prepared by Sherman et al. (1979), as was YPD media. MMS was purchased from Eastman Kodak Co. (Rochester, NY), and yeast α -factor, 4-NQO, and HU were purchased from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, T4 polymerase, and DNA ligase were purchased from New England Biolabs and used under the conditions suggested by the supplier.

Isolation of the *RNR1* and *RNR3* genes using low-stringency hybridization

A λ EMBL3A library of yeast genomic DNA was probed at reduced stringency with a ³²P-labeled 3.0-kb *Bam*HI fragment of the mouse M1 cDNA as a probe. Filters were prepared by the method of Benton and Davis (1977) and hybridized to denatured probe (10⁶ cpm/ml) in 5 × SSPE, 0.1% SDS, 5 × Denhart's solution, and 100 μ g/ml denatured salmon sperm DNA at 42°C overnight without formamide. Filters were washed twice with 1 liter of 5 × SSPE and 0.1% SDS at 42°C and exposed to film while wet. This was repeated at 52°C and 62°C, taking special care not to let the filters dry out. Ten plaques that retained signal at the 62°C wash were purified for further analysis. DNA was prepared from these phage, and Southern analysis was performed to determine that each phage contained DNA that would cross-hybridize with the M1 probe. Phage fell into two classes that were based on restriction maps as described in the text.

DNA sequencing

Plasmid DNAs were sequenced by the method of Sanger et al. (1977). Single-stranded plasmid DNAs were prepared by the method of Zagursky and Berman (1984), by use of R408 (Russell et al. 1986) as a helper phage.

Strains and plasmids

Yeast strains used in these experiments are presented in Table 3. The *E. coli* strain used as a host for constructions and plasmid amplification was JM107. pRS46 is a 2- μ m-based *TRP1* vector provided by R. Sikorfki and J. Shero. p215 is a clone of the chromosomal *SUP11* locus, and p330 is a clone of the chromosomal *TRP1* gene, both provided by P. Hieter (unpubl.). The pUN vectors were described previously (Elledge and Davis 1988).

Plasmids constructed for this study

The *RNR1* and *RNR3* genes were subcloned from their respective phage clones, and these subclones were used as sources of DNA for all subsequent manipulations. The *RNR1* gene was subcloned as a 5.9-kb *Kpn*I fragment into *Kpn*I-cleaved pUN10 to create pSE738. The *RNR3* gene was subcloned as a *Sac*I-*Xho*I fragment into *Sac*I-*Xho*I-cleaved pBS KS⁺ (Stratagene, San Diego, CA) to create pSE734. Plasmids containing the gene disruptions were made in the following way. A 2.6-kb *Eco*RI fragment from pSE738 containing the translational start of *RNR1* was subcloned into the *Eco*RI site of pBS KS⁺ to create pSE736. A *Bgl*II-*Bam*HI fragment containing the *URA3* gene was ligated into *Bgl*II-cleaved pSE736 to create pNN446, a *URA3* disruption of the *RNR1* gene. A *Bgl*II fragment containing the *TRP1* gene from p330 was cloned into the *Bam*HI site in pSE734 to create pNN447, a *TRP1* disruption of the *RNR3* gene. pNN448 was created by a triple ligation between the 1.0-kb *Eco*RI-*Kpn*I *SUP11*-containing fragment of p215, the

Table 3. Strains and plasmids used in this study

Strain	Genotype	
YNN402	<i>MATa/MATα ura3-52/ura3-52 LEU2/leu2-3,-112 ADE2/ade2-101</i>	
YNN403	YNN402 <i>RNR1/rnr1 :: URA3</i>	
YNN404	YNN402 <i>RNR3/rnr3 :: TRP1</i>	
YNN405	<i>MATa ura3-52 Δhis3-200 Δtrp1-800 ade2-101 lys2-901</i>	
YNN406	YNN405 pNN448 (<i>RNR1 HIS3</i>)	
YNN407	YNN406 <i>rnr1 :: URA3</i>	
YNN408	YNN407 pNN449 (<i>RNR3 TRP1</i>)	
YNN409	YNN405 <i>rnr1 :: URA3</i> pNN449 (<i>RNR3 TRP1</i>)	
YNN410	<i>MATa ura3-52 Δhis3-200 trp1-289 ade2-101 leu2-3,-112</i>	
YNN411	YNN410 <i>rad9 :: URA3</i>	
RC634	<i>MATa sst1-3 rme1 ade2 his6 met1 ura1 can1 cyh2</i>	
LN114	<i>MATa ura3-52 Δhis3-200 trp1-289 ade2-101</i>	
SX46	<i>MATa ura3-52 his3-832 trp1-289 ade2-101</i>	
Plasmid	Relevant markers	Base plasmid
pNN446	<i>rnr1 :: URA3</i>	pBS KS ⁺
pNN447	<i>rnr3 :: TRP1</i>	pBS KS ⁺
pNN448	<i>RNR1 SUP11 HIS3 CEN4 ARS1</i>	pUN95
pNN449	<i>RNR3 TRP1 2μm</i>	pRS46

5.3-kb *SacI*–*KpnI* *RNR1* fragment from pSE738, and the *EcoRI*–*SacI*-cleaved pUN95 (*HIS3 CEN4 ARS1*) (Elledge and Davis 1988). pNN449 was made by cloning a *SacI*(blunt)–*XhoI* *RNR3*-containing fragment from pSE734 into *KpnI*(blunt)–*SalI* sites of pRS46. The *SacI* and *KpnI* sites were made flush by treatment with T4 DNA polymerase.

Yeast strains constructed in this study

The diploids heterozygous for the *RNR1* and *RNR3* disruptions were made by transplacement of the diploid YNN402. For *RNR1*, YNN402 cells were transformed with the 3.6-kb *EcoRI* fragment from pNN446 containing the *rnr1 :: URA3* disruption, and Ura prototrophs were selected, creating YNN403. For the *RNR3* disruptions, YNN402 cells were transformed with the 6.7-kb *SacI*–*XhoI* fragment from pNN447 containing the *rnr3 :: TRP1* disruption, and Trp prototrophs were selected, creating YNN404. Construction of the sectoring shuffle reporter strain was accomplished by introducing the plasmid pNN448 into YNN405 by transformation and selecting for His prototrophy, creating YNN406. The chromosomal copy of *RNR1* was then disrupted by transforming YNN406 with the 3.6-kb *EcoRI* fragment from pNN446 containing the *rnr1 :: URA3* disruption and selecting for Ura prototrophy, creating YNN407. Transformants were streaked for single colonies on complete minimal media with 0.2× adenine for the determination of sectoring ability. Transplacements that disrupted the plasmid copy of *RNR1* could lose the *HIS3 URA3 SUP11* plasmid in the absence of nutritional selection and would appear sectorial. However, transplacement events that disrupted the chromosomal copy of *RNR1* would not produce red sectors because the *SUP11* gene is linked to the only viable copy of *RNR1* on the plasmid, and every plasmid loss event that would produce red sectors leads to cell death. Transformants that appeared homogeneously white on this media were chosen for further study. They were tested for growth on YP glycerol plates to eliminate petite mutants, which also appear homogeneously white. None of the transformants tested were petite mutants. Several strains were analyzed by Southern analysis and shown to have disrupted the chromosomal *RNR1* gene, and one was chosen for further analysis. This strain, YNN407, was

then transformed with pNN449 (*RNR3 TRP1 2 μm*) by selecting for Trp prototrophy. The resulting strain, YNN408, was tested for sectoring ability on minimal glucose medium supplemented with histidine, lysine, and 0.2× adenine. It was capable of losing pNN448 and producing sectors, as shown in Figure 3. A red colony that had lost pNN448 was isolated, YNN409, and shown by Southern analysis to have lost the functional *RNR1* allele linked to the plasmid.

DNA blot and RNA blot analysis

DNA was labeled by the hexamer primer method (Feinberg and Vogelstein 1983). Hybridizations for Southern blots were carried out, as described previously (Elledge and Davis 1987). RNA was resolved on formaldehyde–1% agarose gels (Maniatis et al. 1982), and hybridizations were carried out as described for the Southern analysis above.

Induction of RNR expression of 4-NQO

SK46 cells were grown to mid-log phase in YPD. 4-NQO was added to a final concentration of 0.25 μg/ml. Aliquots of cells were taken at 30-min intervals for RNA preparation and analysis. Probes for the RNA blot were the 1.0-kb *HindIII*–*SmaI* fragment of *URA3*, the 1.7-kb *BglII*–*EcoRI* fragment of *RNR1*, and the 1.1-kb *BamHI*–*EcoRI* fragment of *RNR3*.

HU and MMS induction during cell-cycle arrest

The strain RC634 was examined to determine whether the stage of the cell-cycle affects the DNA damage induction of the *RNR* genes. RC634 contains the *sst1* mutation that eliminates a protease that normally degrades α-factor. Consequently this strain is much more sensitive to the presence of α-factor. Yeast α-factor causes the arrest of *MATa* cells at the G₁ stage of the cell cycle (Buckling-Throm et al. 1973). We grew RC634 in YPD to OD₆₀₀ of 0.3 and then added α-factor directly to the media at a concentration of 10 μM. After 2.5 hr, these cells were monitored for schmooring, the characteristic elongated morphology that indicates the α-factor commitment to mating. Greater than 99% of the cells exhibited such morphology at this point.

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Then, either HU was added to a final concentration of 100 mM or MMS was added to a final concentration of 0.01% (vol/vol), and cells were incubated for an additional 4 hr. Cells that were not exposed to α -factor were also treated with HU or MMS as cycling controls. Cells treated with α -factor but not HU or MMS were monitored after 4 hr for the appearance of buds. Although they maintained a rather exaggerated elongated morphology, 99% of the cells remained unbudded. After 4 hr, cells were harvested, and RNA was prepared for Northern analysis as described previously (Elledge and Davis 1987). The probes for the RNA blot were the internal *Hind*III–*Sma*I fragment of *URA3*, the 1.7-kb *Bgl*II–*Eco*RI fragment of *RNR1*, and the 1.1-kb *Bam*HI–*Eco*RI fragment of *RNR3*.

Cell-cycle synchronization

To determine whether the *RNR1* and *RNR2* transcripts were regulated by the cell cycle, LN114 was grown to OD₆₀₀ of 0.5 in 1 liter of YPD at pH 3.9 (correct pH was achieved with HCl) and α -factor was added to a final concentration of 1.6 μ g/ml. Cells were allowed to synchronize for 1 hr, at which time an additional 0.8 μ g/ml of α -factor was added. When ~90% of the cells appeared unbudded, ~2.25 hr from the first α -factor addition, cells were spun down and washed twice with YPD of normal pH and resuspended in 800 ml of YPD. Forty-milliliter aliquots of cells were taken at 10-min time points for RNA preparation and analysis. RNA blots were prepared as described previously. Probes used were the *Hind*III–*Sma*I fragment of *URA3*, the internal *Eco*RI–*Sal*I fragment of *RNR2*, the 1.7-kb *Bgl*II–*Eco*RI fragment of *RNR1*, and the 3.0-kb *Sal*I fragment of the *POL1* gene.

For determination of the dependence of cell-cycle induction of *RNR1* message on protein synthesis, cells were blocked in G₁ with α -factor, as described above, with the exception that cells were initially treated with 2 μ g/ml α -factor and after 75 min of incubation, an additional 1 μ g/ml was added. When >95% schmoos were observed, the culture was split; sample 1 was left untreated, whereas sample 2 was made 100 μ g/ml cycloheximide. After 10 min, the cells were spun down, washed twice with and resuspended in YPD for sample 1 and YPD plus 100 μ g/ml cycloheximide for sample 2. Time points were taken at 0, 20, 50, and 80 min for RNA preparation and analysis.

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Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase.

S J Elledge and R W Davis

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