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

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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 MATα 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]

Received December 19, 1989; revised version accepted February 12, 1990.

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 Saccharomyces 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-
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 (Schumberi 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, CACGAg, 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 α2β2. 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 G0 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 BamHI 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] BamHI; [Bgl] BglII; [R1] EcoRI; [H3] HindIII; [Kpn] KpnI; [Sac] SacI; [Sal] SalI; [Xho] XhoI. TheURA3 and TRP1 genes shown above the RNR genes show the sizes and positions of insertions used to create the chromosomal disruptions mtn1 : URA3 and mtn3 : 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.

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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, m1 :: URA3 and m3 :: 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 diploids heterozygous for respective rr mutations were allowed to undergo sporulation, and tetrad analysis was performed (Table 1). Of the 27 tetrads analyzed for the m1 :: 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 m3 :: 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 m3 :: TRP1 haploids did not appear to have a growth defect relative to their RNR+ sister spore clones.

The RNR1 gene encodes a functional regulatory subunit of ribonucleotide reductase. To search further for a function for RNR1, the RNR3 gene was placed on the TRP1-containing 2-μm 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 m1 :: 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.
Table 1. Tetrad analysis of RNR1 and RNR3 mutants

| Strain | Number of viable spores per tetrad | Number of tetrads observed | Genotype |
|--------|-----------------------------------|-----------------------------|----------|
| YNN403 | 4                                 | 0                           | ura3     |
|        | 3                                 | 0                           | 0        |
|        | 2                                 | 25                          | 0        |
|        | 1                                 | 2                           | 0        |
| YNN404 | 4                                 | 29                          | 58       |
|        | 3                                 | 3                           | 4        |
|        | 2                                 | 0                           | 0        |
|        | 1                                 | 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.

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 POL1, URA3, and RNR2 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 G1 to the induction of these transcripts, themselves, is dependent on protein synthesis. To test this hypothesis, cells were arrested in G1 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 G1 phase of the cell cycle with the peptide hormone α-factor (Buckingham-Throm 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 G1-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 surprising 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 G1-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 G1 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 G1-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 damage. For example, treatment with HU or mitomycin C results in a predominantly G2/M arrest with a small proportion of cells arresting in G1. If HU generates a damage signal by stalling DNA replication through a depletion of dNTPs, it would be surprising 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 G1-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 G1 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.

**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 OD600 of 1.0, and 4-NQO was added to a final concentration of 0.25 μg/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.
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-
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 G1 relative 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 G0 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-

| Cell-cycle stage | YNN410 (RAD+) cells [%] | YNN411 (rad9::URA3) cells [%] |
|------------------|-------------------------|-----------------------------|
| Unbudded         | -HU: 22 +HU: 2          | -HU: 24 +HU: 1              |
| Small buds       | -HU: 27 +HU: 1          | -HU: 28 +HU: 1              |
| Large buds       | -HU: 51 +HU: 97         | -HU: 48 +HU: 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.
highly homologous to the S. cerevisiae. CDC28.

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 requires 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 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.

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 G2 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. Selective 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 λEML3A library of yeast genomic DNA was probed at reduced stringency with a 32P-labeled 3.0-kb BamHI 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 (10 6 cpm/ml) in 5 x SSPE, 0.1% SDS, 5 x Denhart’s solution, and 100 µg/ml denatured salmon sperm DNA at 42°C overnight without formamide. Filters were washed twice with 1 liter of 5 x 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 define 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 (Russel 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-μm-based TRPl 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 TRPl 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 KpnI fragment into KpnI-cleaved pUN10 to create pSE738. The RNR3 gene was subcloned as a SacI–XhoI fragment into SacI–XhoI-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 EcoRI fragment from pSE738 containing the translational start of RNR1 was subcloned into the EcoRI site of pBS KS+ to create pSE736. A BglII–BamHI fragment containing the URA3 gene was ligated into BglII-cleaved pSE736 to create pNN446, a URA3 disruption of the RNR1 gene. A BglII fragment containing the TRPl gene from p330 was cloned into the BamHI site in pSE734 to create pNN447, a TRPl disruption of the RNR3 gene. pNN448 was created by a triple ligation between the 1.0-kb EcoRI–KpnI SUP11-containing fragment of p215, the
Table 3. Strains and plasmids used in this study

| Strain   | Genotype                                      |
|----------|-----------------------------------------------|
| YNN402   | MATa/MATa ura3-52/ura3-52 LEU2/leu2-3,-112 ADE2/ade2-101 |
| YNN403   | YNN402 RNR1/rn1 :: URA3                       |
| YNN404   | YNN402 RNR3/rn3 :: TRP1                       |
| YNN405   | YNN402 ura3-52 AΔhis3-200 Δtrpl-800 ade2-101 lys2-901   |
| YNN406   | YNN405 pNN448 [RNR1 HIS3]                     |
| YNN407   | YNN406 rn1 :: URA3                            |
| YNN408   | YNN407 pNN449 [RNR3 TRP1]                     |
| YNN409   | YNN405 rn1 :: URA3 pNN449 [RNR3 TRP1]         |
| YNN410   | YNN409 rn3 :: URA3                            |
| YNN411   | YNN410 rad9 :: URA3                           |
| RC634    | YNN410 sst1-3 rrn1 ade2 his6 met1 ura1 can1 cyh2 |
| LN114    | YNN410 ura3-52 AΔhis3-200 trpl-800 ade2-101     |
| SX46     | YNN410 ura3-52 his3-832 trpl-800 ade2-101       |

| Plasmid | Relevant markers | Base plasmid |
|---------|------------------|--------------|
| pNN446  | rn1 :: URA3      | pBS KS+      |
| pNN447  | rn3 :: TRP1      | pBS KS+      |
| pNN448  | RNR1 SUP11 HIS3 CEN4 ARS1 | pUN95       |
| pNN449  | RNR3 TRP1 2μm    | pRS46        |

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 rn1 :: 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 rm3 :: 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 rn1 :: URA3 disruption and selecting for Ura prototrophy, creating YNN407. Transformants were streaked for single colonies on complete minimal media with 0.2 x 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 sectored. 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 hybridization 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 x 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 RsII–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 [Bucking-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 schmooing, the characteristic elongated morphology that indicates the α-factor commitment to mating. Greater than 99% of the cells exhibited such morphology at this point.
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 a-factor were also treated with HU or MMS as cycling controls. Cells treated with a-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 HindIII-Smal fragment of URA3, the 1.7-kb BglII-EcoRI fragment of RNR1, and the 1.1-kb BamHI-EcoRI 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 a-factor was added to a final concentration of 1.6 µg/ml. Cells were allowed to synchronize for 1 hr, at which time an additional 0.8 µg/ml of a-factor was added. When ~90% of the cells appeared unbudded, ~2.25 hr after the first a-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 HindIII-Smal fragment of URA3, the internal EcoRI-SalI fragment of RNR2, the 1.7-kb BglII-EcoRI fragment of RNR1, and the 3.0-kb SalI fragment of the POL1 gene.

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

Acknowledgments

We thank W. Harper and D. Allis for critical comments on the manuscript; L. Thelander and D. Martin for plasmids, B. Fuller for strains, and W. Shanabruch and A. Sachs for helpful discussions. We also thank K. McIntee and Kevin Yagle for communicating unpublished results. SJE was a Helen Hay Whitney Fellow and a National Institutes of Health grant AG02908 to R.W.D.

References

Albert, D.A. and E. Nordzenski. 1989. M2 subunit of ribonucleotide reductase is a target of cyclic AMP-dependent protein kinase. J. Cell. Physiol. 138: 129–136.

Andrews, B.J. and I. Herskowitz. 1989. Identification of a DNA-binding factor involved in cell cycle control of the yeast HO gene. Cell 57: 21–29.

Barker, D.G., J.M. White, and L.H. Johnston. 1985. The nucleotide sequence of the DNA ligase gene (CDC 9) from Saccharomyces cerevisiae: A gene which is cell-cycle regulated and induced in response to DNA damage. Nucleic Acids Res. 13: 8323–8337.

Benton, W.D. and R.W. Davis. 1977. Screening λgt recombinant clones by hybridization to recombinant clones in situ. Science 196: 180–183.

Buckling-Throm, E., W. Duntze, L.H. Hartwell, and T. Manney. 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. 76: 99–110.

Burns, V.W. 1956. X-ray induced division delay of individual yeast cell. Radiat. Res. 4: 394–412.

Caras, I.W., B.B. Levinson, M. Fabry, S.R. Williams, and D.W. Martin. 1985. Cloned mouse ribonucleotide reductase subunit M1 cDNA reveals amino acid sequence homology with Escherichia coli and herpesvirus ribonucleotide reductase. J. Biol. Chem. 260: 7015–7022.

Courchesne, W.E., R. Kunisawa, and J. Thorner. 1989. A putative protein kinase overcomes pheromone-induced arrest of cell cycling in S. cerevisiae. Cell 58: 1107–1119.

Cross, F.R. 1988. DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of Saccharomyces cerevisiae. Mol. Cell. Biol. 8: 4675–4684.

Elledge, S.J. and R.W. Davis. 1987. Identification and isolation of the gene encoding the small subunit of ribonucleotide reductase from Saccharomyces cerevisiae: A DNA damage-inducible gene required for mitotic viability. Mol. Cell. Biol. 7: 2783–2793.

——. 1988. A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in Saccharomyces cerevisiae. Gene 70: 303–312.

——. 1989a. DNA-damage induction of ribonucleotide reductase. Mol. Cell. Biol. 9: 4932–4949.

——. 1989b. Identification of a damage regulatory element of RNR2, and evidence that four distinct proteins bind to it. Mol. Cell. Biol. 9: 5373–5386.

Engstrom, Y., S. Eriksson, I. Jildvik, S. Skog, L. Thelander, and B. Tribukait. 1983. Cell cycle-dependent expression of mammalian ribonucleotide reductase. J. Biol. Chem. 260: 9114–9116.

Evans, T., E.T. Rosenthal, J. Tourblom, D. Distel, and T. Hunt. 1983. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33: 389–396.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeled DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6–13.

Hadjivassiliou, J.A., C. Wittenberg, H.E. Richardson, M. Lopes, and S.I. Reed. 1989. A family of cyclin homologs that control the G1 phase in yeast. Proc. Natl. Acad. Sci. 86: 6255–6259.

Hereford, L. and L.H. Hartwell. 1974. Sequential gene function in the initiation of Saccharomyces cerevisiae DNA synthesis. J. Mol. Biol. 84: 445–461.

Hereford, L.M., M.A. Osley, J.R. Ludwig, and C.S. McGlaughlin. 1981. Cell-cycle regulation of yeast histone mRNA. Cell 24: 367–375.

Hurd, H. and J. Roberts. 1989. Upstream regulatory sequences of the yeast RNR2 gene include a repression sequence and an activation site that binds the RAPI protein. Mol. Cell. Biol. 9: 5361–5372.

Hurd, H., C.W. Roberts, and J.W. Roberts. 1987. Identification of the gene for the yeast ribonucleotide reductase small subunit and its inducibility by methyl methanesulfonate. Mol. Cell. Biol. 7: 3673–3677.

Johnston, L.H., J.H.M. White, A.L. Johnson, G. Lucchini, and P. Pleviani. 1987. The yeast DNA polymerase I transcript is regulated in both the mitotic cell cycle and in meiosis and is
also induced after DNA damage. *Nucleic Acids Res.* **15**: 5017–5030.

Kupiec, M. and G. Simchen. 1986. Regulation of the RAD6 gene of *Saccharomyces cerevisiae* in the mitotic cell cycle and in meiosis. *Mol. Gen. Genet.* **203**: 538–543.

Lammers, M. and H. Follman. 1984. Deoxyribonucleotide biosynthesis in yeast. *Eur. J. Biochem.* **140**: 281–287.

Lowden, M. and E. Vitols. 1973. Ribonucleotide reductase activity during the cell cycle of *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **158**: 177–184.

Maniatis, T., E.F. Fritsch, and E.F. Sambrook. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Mann, G.J., E.A. Musgrove, R.M. Fox, and L. Thelander. 1988. Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation. *Cancer Res.* **48**: 5151–5156.

McIntosh, E.M., R.W. Ord, and R.K. Storms. 1988. Transcriptional regulation of the cell cycle-dependent thymidylate synthesis gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**: 4616–4624.

Nash, R., J. Tokiwa, S. Anand, K. Erikson, and B. Futcher. 1988. The Whi1 gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7**: 4335–4346.

Nasmyth, K. 1985. A repetitive DNA sequence that confers cell-cycle START (CDC28)-dependent transcription of the HO gene. *Cell* **42**: 225–235.

———. 1987. The determination of mother cell-specific mating type switching in yeast by a specific regulator of HO transcription. *EMBO J.* **6**: 243–248.

Osley, M.A. and D. Lycans. 1987. trans-acting regulatory mutations that alter transcription of *Saccharomyces cerevisiae* histone genes. *Mol. Cell. Biol.* **7**: 4204–4210.

Osley, M.A., J. Gould, S. Kim, M. Kane, and L. Hereford. 1986. Identification of sequences in a yeast histone promoter involved in periodic transcription. *Cell* **45**: 537–544.

Peterson, T.A., L. Prakash, S. Prakash, M.A. Osley, and S.I. Reed. 1985. Regulation of CDC9, the *Saccharomyces cerevisiae* gene that encodes DNA ligase. *Mol. Cell. Biol.* **5**: 226–235.

Pringle, J.R. and L.H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In *The molecular biology of the yeast Saccharomyces cerevisiae*. *Life cycle and inheritance* (ed. J.N. Strathern, E.W. Jones, and J.R. Broach), pp. 97–142. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Rothstein, R. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101**: 202–211.

Ruby, S.W. and J.W. Szostack. 1985. Specific *Saccharomyces cerevisiae* genes are expressed in response to DNA-damaging agents. *Mol. Cell. Biol.* **5**: 75–84.

Russel, M., S. Kidd, and M.R. Kelly. 1986. An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene* **45**: 333–338.

Sanger, F., F. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.

Schiestl, R.H., P. Reynolds, S. Prakash, and L. Prakash. 1989. Cloning and sequence analysis of the *Saccharomyces cerevisiae* RAD9 gene and further evidence that its product is required for cell cycle arrest induced by DNA damage. *Mol. Cell. Biol.* **9**: 1882–1896.

Schumberli, D. 1986. Cell-cycle regulation of histone gene expression. *Cell* **45**: 471–472.

Sherman, F., G.R. Fink, and C.W. Lawrence. 1979. *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Slater, M.L. 1973. Effect of reversible inhibition of deoxyribonucleic acid synthesis on the yeast cell cycle. *J. Bacteriol.* **113**: 263–270.

Storms, R.K., R.W. Ord, M.T. Greenwood, B. Mirdamadi, F.K. Chu, and M. Belfort. 1984. Cell-cycle dependent expression of thymidylate synthase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**: 2858–2864.

Thelander, L. and F. Berg. 1986. Isolation and characterization of expressible cDNA clones encoding the M1 and M2 subunits of mouse ribonucleotide reductase. *Mol. Cell. Biol.* **6**: 3433–3442.

Weinert, T.A. and L.H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241**: 317–322.

———. 1989. Controls that ensure the order of cell cycle events. *Science* **246**: 629–634.

White, J., S.R. Green, D.G. Barker, L.B. Dumas, and L.H. Johnston. 1988. The CDC8 transcript is cell cycle regulated in yeast and is expressed coordinately with CDC9 and CDC21 at a point preceding histone transcription. *Exp. Cell Res.* **171**: 223–231.

Zagursky, R.J. and M.L. Berman. 1984. Cloning vectors that yield high levels of single-stranded DNA for rapid sequencing. *Gene* **27**: 183–191.
Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase.

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*Genes Dev.* 1990, 4:
Access the most recent version at doi:10.1101/gad.4.5.740