Constitutively high dNTP concentration inhibits cell cycle progression and the DNA damage checkpoint in yeast *Saccharomyces cerevisiae*

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In eukaryotic cells the concentration of dNTP is highest in S phase and lowest in G_1 phase and is controlled by ribonucleotide reductase (RNR). RNR activity is eliminated in all eukaryotes in G_1 phase by a variety of mechanisms: transcriptional regulation, small inhibitory proteins, and protein degradation. After activation of RNR upon commitment to S phase, dATP feedback inhibition ensures that the dNTP concentration does not exceed a certain maximal level. It is not apparent why limitation of dNTP concentration is necessary in G_1 phase. In principle, dATP feedback inhibition should be sufficient to couple dNTP production to utilization. We demonstrate that in *Saccharomyces cerevisiae* constitutively high dNTP concentration transiently arrests cell cycle progression in late G_1 phase, affects activation of origins of replication, and inhibits the DNA damage checkpoint. We propose that fluctuation of dNTP concentration controls cell cycle progression and the initiation of DNA replication.

DNA replication | ribonucleotide reductase

The concentration of dNTP, the precursors for DNA synthesis, fluctuates during the eukaryotic cell cycle because of changes in the activity of ribonucleotide reductase (RNR). This enzyme catalyzes the rate-limiting step in the biosynthesis of all four dNTPs and is regulated by multiple mechanisms (1–4). The enzyme's allosteric activity site controls the concentration of dNTP: when [dNTP] reach a certain level, the RNR activity is down-regulated by dATP feedback inhibition (5).

In addition to the allosteric control, RNR activity is tightly cell cycle-regulated and is restricted to S, G_2, and M phases (1, 6). In eukaryotes RNR consists of a large and a small subunit, both necessary for catalysis. In mammalian cells elimination of RNR activity in G_1 is achieved by anaphase-promoting complex Cdh1-dependent degradation of the small RNR subunit in mitosis and by the transcriptional repression of the small RNR subunit gene in G_1 phase (7, 8). In addition to transcriptional regulation, in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* RNR activity is controlled by small proteins called Sml1 and Spd1, respectively, that bind to RNR in G_1 phase and inhibit its activity (9–11). Sml1 and Spd1 are degraded upon entry into S phase or in response to DNA damage (9, 12). Finally, in both yeasts the RNR activity is reported to be controlled by differential localization of its subunits during the cell cycle and after DNA damage (9, 13–15).

The significance of the elimination of RNR activity in G_1 phase is not clear, especially in cycling yeast cells, where G_1 phase is short. The dATP feedback inhibition would restrict the RNR activity and therefore couple dNTP concentration to utilization, even if a fully active enzyme were present in G_1 phase. The consumption of dNTP during G_1 phase is minimal and is limited to mitochondrial DNA synthesis and repair. The deoxyribonucleosides produced by 5′-nucleotidases in mammalian cells are able to penetrate the cellular membrane and can be excreted (1), but no degradation of excess deoxyribonucleotides to deoxyribonucleosides or excretion is documented in yeast. Yeast, unlike mammalian cells, also lacks deoxynucleoside kinases, which together with deoxynucleotidases form a substrate cycle important for regulation of [dTTP] (1, 16). It is usually assumed that elimination of RNR activity ensures that unscheduled DNA replication does not occur in G_1, but there are no data supporting this notion.

To understand the role of [dNTP] fluctuation during the cell cycle, we chose *S. cerevisiae* as a model organism, where both cell cycle and RNR biology are well studied. In *S. cerevisiae* [dNTP] increases in response to DNA damage up to 8-fold above [dNTP] of a logarithmically growing culture (17). This increase is mediated by the Mec1/Rad53 DNA damage checkpoint, which activates transcription of the RNR genes and promotes degradation of the inhibitory protein Sml1 (11, 18, 19).

The increase in [dNTP] in *S. cerevisiae* during DNA damage is directly correlated to DNA damage tolerance (17). In the *rnr1-D57N* strain, in which the dATP feedback inhibition of RNR is nonfunctional, [dNTP] transiently increases in response to DNA damage ≈30-fold. The ability of the *rnr1-D57N* mutant to increase [dNTP] above wild-type levels in response to DNA damage is associated with an up to 500-fold higher tolerance of DNA damage (17). It is not known whether a constant presence of an ≈30-fold higher [dNTP] during the cell cycle also increases DNA damage survival.

To study the effect of continuously high [dNTP] on the DNA damage checkpoint and cell cycle progression, we introduced into the yeast genome an additional *RNR1* or *rnr1-D57N* allele, both under the regulation of the inducible *GAL1* promoter. Expression of the *GAL1*-driven *rnr1-D57N* allele resulted in an ≈35-fold increase in [dNTP], similar to the increase in the *rnr1-D57N* strain during a DNA damage response, whereas expression of the *GAL1*-driven wild-type *RNR1* resulted in an ≈10-fold increase in [dNTP], similar to the increase in a wild-type strain during DNA damage response. The continuous overexpression of *rnr1-D57N* delayed cell cycle progression, particularly entry into S phase, delayed activation of prereplicative complexes (pre-RCs) at origins of DNA replication, and also resulted in DNA damage sensitivity due to a defect in the DNA damage checkpoint response. When the number of pre-RCs was reduced by mutations in the origin recognition complex, cells were sensitive to overexpression of wild-type *RNR1*. These results suggest that [dNTP] controls cell cycle progression by regulation of pre-RC utilization during normal growth and during a response to DNA damage.

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Abbreviations: RNR, ribonucleotide reductase; pre-RC, prereplicative complex; gal, galactose; MMS, methyl methanesulfonate; pre-IC, preinitiation complex; TMPK, thymidylate kinase; YPD, 1% yeast extract/2% peptone/2% dextrose; YPGal, 1% yeast extract/2% peptone/2% gal; YPRaf, 1% yeast extract/2% peptone/2% raffinose.

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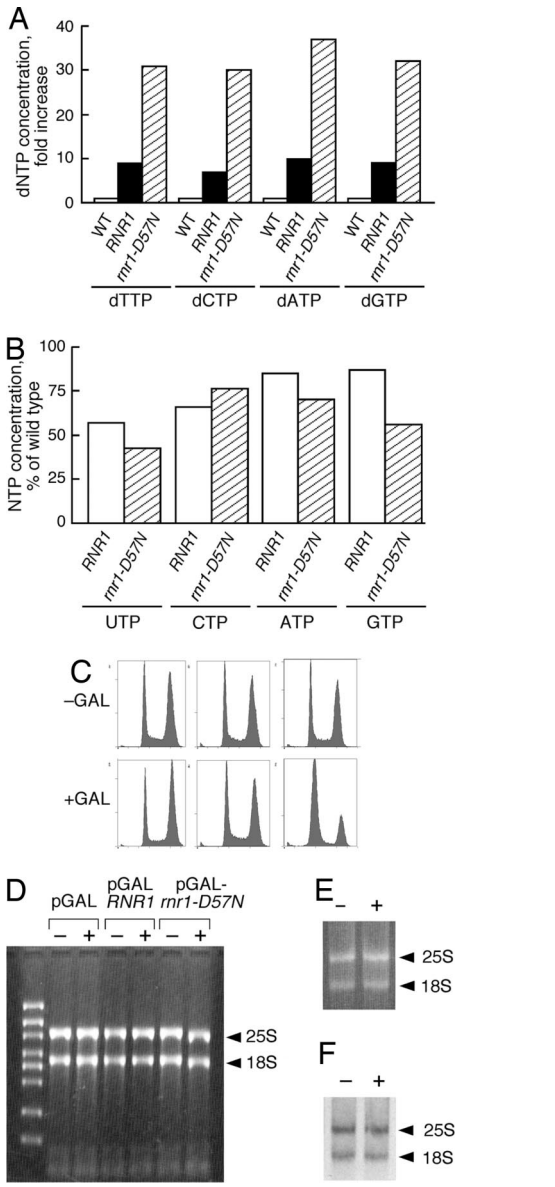

Fig. 1. Expression of *RNR1* alleles increases [dNTP] and decreases [NTP] but does not affect transcription. (*A*) Changes in [dNTP] in pGAL-*RNR1* and pGAL-*rnr1-D57N* strains after a 3-h gal induction. [dNTP] in the wild-type W1588-4C strain was assigned a value of 1. (*B*) [NTP] in pGAL-*RNR1* (AC438) and pGAL-*rnr1-D57N* (AC439) strains after a 3-h gal induction, in percentage of [NTP] in a wild-type strain. (*C–F*) pGAL (AC437), pGAL-*RNR1* (AC438), and pGAL-*rnr1-D57N* (AC439) strains were grown in minimal media without methionine until an OD_{600} of 0.2 and after the addition of 2% gal or raf incubated for 4 h. (*C*) Flow-cytometric analysis of pGAL (*Left*), pGAL-*RNR1* (*Center*), and pGAL-*rnr1-D57N* (*Right*). (*D*) Ethidium bromide staining showing equal amounts of 25S and 18S rRNA. (*E* and *F*) Ethidium bromide staining (*E*) and corresponding autoradiography (*F*) of the [^{3}H]methionine-labeled rRNA sample from the pGAL-*rnr1-D57N* (AC439) strain. + and − indicate presence and absence of gal.

Results

Expression of *RNR1* Alleles Increases [dNTP] in the Absence of DNA Damage. The *GAL1*-regulated *RNR1* or *rnr1-D57N* alleles were integrated at the *URA3* locus of a wild-type W1588-4C *S. cerevisiae* strain. After 3 h of induction by galactose (gal), [dNTP] proportionally increased ≈9–10 times in the pGAL-*RNR1* strain and ≈35 times in the pGAL-*rnr1-D57N* strain (Fig. 1*A*). Induction of both strains also resulted in a decrease in [NTP], a 1.2- to 1.8-fold decrease in the pGAL-*RNR1* strain, and a 1.3- to 2.2-fold increase in the pGAL-*rnr1-D57N* strain (Fig. 1*B*). The same changes in [dNTP] and [NTP] were found in both strains growing logarithmically in gal-containing media for more than six doubling times (data not shown). Thus, expression of *RNR1* alleles results in a sustained increase in dNTP concentration even in the absence of DNA damage. The increases in [dNTP] in pGAL-*RNR1* and pGAL-*rnr1-D57N* were of the same magnitude as in the DNA-damage-treated wild-type and *rnr1-D57N* strains (where *rnr1-D57N* is under its own promoter), respectively (17).

Expression of *rnr1-D57N* Causes Slow Proliferation but Does Not Activate the DNA Damage Checkpoint. Whereas the expression of *RNR1* had no effect on the proliferation rate of yeast, the expression of *rnr1-D57N* slowed it down. The plating efficiency was, however, the same among the strains with a pGAL promoter only, pGAL-*RNR1*, or pGAL-*rnr1-D57N* on YPD and YPGal (data not shown and Fig. 2*A*), demonstrating that the overexpression of the Rnr1-D57N is not toxic. Gal induction slowed proliferation of the pGAL-*rnr1-D57N* strain (Fig. 2*A*), and flow cytometry showed an enrichment of cells in G_1 phase or at the G_1/S transition, but most notably a reduction of the number of cells in S phase (Fig. 1*C*). This change in cell cycle distribution did not occur when *RNR1* was induced (Fig. 1*C*).

It is possible that the slow proliferation of the gal-induced pGAL-*rnr1-D57N* strain is caused by the ≈2-fold decrease in [NTP]. In this case the rate of transcription in the pGAL-*rnr1-D57N* strain should be reduced after induction. To test this possibility, we compared the rate of synthesis of rRNA in the pGAL, pGAL-*RNR1*, and pGAL-*rnr1-D57N* strains with and without induction. There was no difference in the amount of rRNA or rRNA synthesis rate in the pGAL-*rnr1-D57N* strain with and without induction (Fig. 1 *D–F*). These results suggest that it is not the depletion of NTP that caused the slow proliferation of the pGAL-*rnr1-D57N* strain by blocking transcription.

Hydroxyurea inhibits RNR activity and depletes the dNTP pool, causing activation of the Mec1/Rad53 checkpoint and cell cycle arrest in early S phase (20). It is possible that the unusually high [dNTP], like the unusually low [dNTP] caused by hydroxyurea, interferes with DNA replication by activating the DNA damage checkpoint and arresting cell cycle progression. Activation of the DNA damage checkpoint leads to phosphorylation of the Rad53 protein, resulting in an electrophoretic shift of the Rad53 (21, 22). The activated Mec1/Rad53 checkpoint also induces transcription of all RNR genes, notably *RNR3* (19). Neither Rad53 phosphorylation (Fig. 3*A*) nor an increase of Rnr3 (Fig. 3*B*) was observed in the gal-induced pGAL-*rnr1-D57N* strain, suggesting that the slower rate of S-phase progression was not due to activation by high [dNTP] of the known intra-S-phase DNA damage checkpoint pathway.

Continuous Expression of *rnr1-D57N* Inhibits the DNA Damage Checkpoint. An ≈30-fold increase in [dNTP] during DNA damage in a yeast strain with an *rnr1-D57N* allele under its own promoter is associated with an increased DNA damage tolerance (17). In contrast, the pGAL-*rnr1-D57N* strain, although it also increases [dNTP] ≈35-fold when grown on gal, is DNA-damage-sensitive (Fig. 2*A*). Thus, such a high [dNTP] is deleterious when present continuously, but a transient induction of the same [dNTP] is beneficial for DNA damage survival. The pGAL-*RNR1* strain was more resistant to DNA damage on YPGal compared with wild type (Fig. 2*A*) and approximately as resistant to DNA damage as a strain with an *rnr1-D57N* allele under its own promoter (17).

Consistent with the DNA-damage-sensitive phenotype of the pGAL-*rnr1-D57N* strain on YPGal, Rad53 was not activated, and the cell cycle was not arrested by DNA-damaging agents (Fig. 2 *B* and *C*). In the presence of methyl methanesulfonate (MMS), only a small percentage of the gal-induced pGAL-*rnr1-D57N* cells slowed down S-phase progression, whereas the bulk of the cells

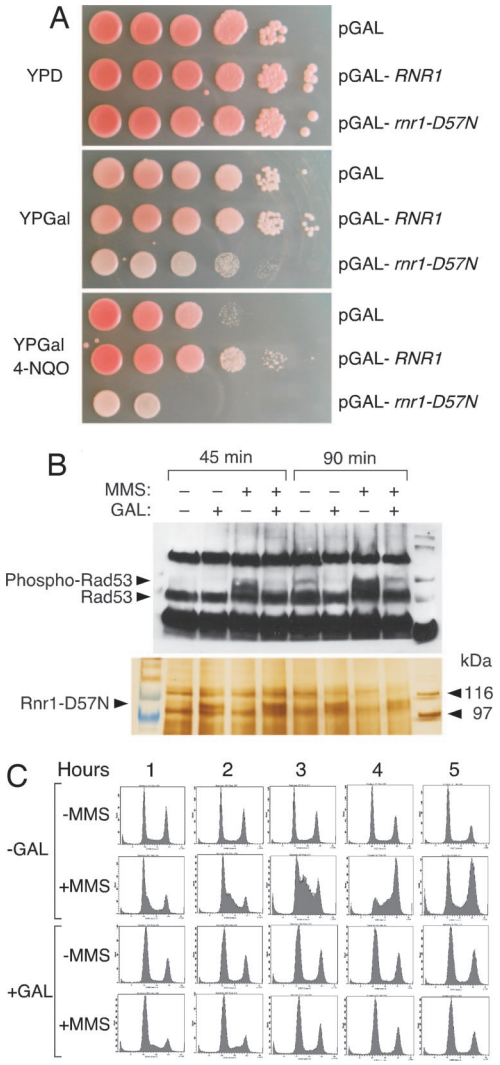

Fig. 2. Expression of pGAL-*rnr1-D57N* leads to DNA damage sensitivity and defects in DNA damage checkpoint. (*A*) Overnight cultures of pGAL (AC437), pGAL-*RNR1* (AC438), and pGAL-*rnr1-D57N* (AC439) strains grown in YPD were spotted at 10-fold serial dilutions on YPD, YPGal, and YPGal/0.3 mg/4-nitroquinoline 1-oxide plates. (*B*) pGAL-*rnr1-D57N* (AC439) strain was grown in YPRaf to an OD_{600} of 0.2, and half of the culture was induced by 2% gal for 2 h (indicated by +). After that, 0.01% MMS was added (indicated by +), and samples were collected for analysis by Western blotting (*Upper*) and SDS/PAGE (*Lower*) at 45 and 90 min. (*C*) pGAL-*rnr1-D57N* (AC439) strain was grown in YPRaf to an OD_{600} of 0.2, and half of the culture was induced by 2% gal for 2 h (indicated by GAL). After that, 0.01% MMS was added (indicated by +MMS), and samples were collected for flow-cytometric analysis.

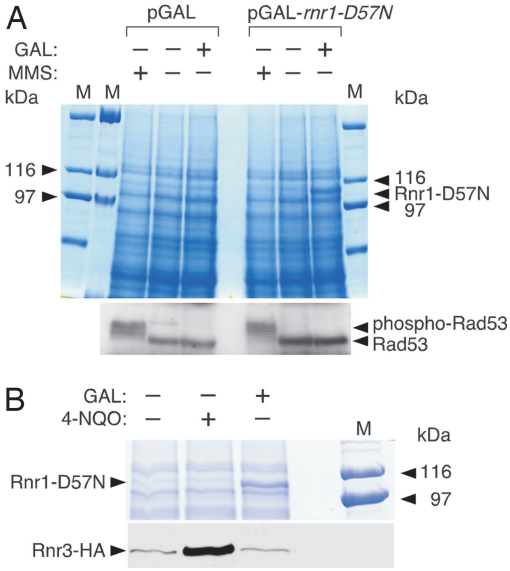

Fig. 3. Expression of pGAL-*rnr1-D57N* does not activate Rad53 and does not induce transcription of *RNR3*. (*A*) pGAL (AC437) and pGAL-*rnr1-D57N* (AC439) strains were grown in YPRaf to an OD_{600} of 0.2, and, after the addition of either 0.01% MMS, 2% gal, or no addition of drug, they were incubated for 3 h. The expression of Rnr1-D57N was analyzed by SDS/PAGE (*Upper*) and Rad53 phosphorylation by Western blotting (*Lower*). M, protein marker lanes. (*B*) pGAL-*rnr1-D57N RNR3-HA* (AC454-4F) strain was grown in liquid YPRaf to an OD_{600} of 0.2, and, after the addition of either 0.3 mg of 4-nitroquinoline 1-oxide, 2% gal, or no addition of drug, they were incubated for 3 h. The expression of Rnr1-D57N was analyzed by SDS/PAGE (*Upper*), and the expression of Rnr3-HA was analyzed by Western blotting (*Lower*).

continued to slowly progress through the cell cycle (the OD of the culture continued to increase slowly during the 5-h time course). In the same strain, in the absence of high [dNTP], MMS induced the checkpoint and cells progressed very slowly through S phase (Fig. 2 *B* and *C*).

Expression of *rnr1-D57N* from GAL1 Promoter Reduces Cdc45 Loading onto Chromatin. High [dNTP] slows down progression into S phase. This may be because of inefficient assembly of pre-RCs at origins of DNA replication that normally occurs in G_1 phase, when [dNTP] normally is low. Alternatively, pre-RC assembly might be normal, but activation of these licensed origins might be inefficient in the presence of high [dNTP].

To test these contrasting ideas, the pGAL-*rnr1-D57N* strain was synchronized either in late G_1 phase by α factor or in mitosis using a *dbf2-1* ts mutation. The α-factor-synchronized cells not induced by gal progressed normally through the cell cycle, whereas the gal-induced cells showed a delayed entry into S phase and a delayed and reduced loading of Cdc45, a preinitiation complex (pre-IC) protein (23), onto chromatin (Fig. 4*A*). The *dbf2-1* pGAL-*rnr1-D57N* cells both induced and not induced by gal exited from mitosis at the same rate, but the gal-induced cells again showed delayed S-phase entry and reduced Cdc45 loading (Fig. 4*B*). Levels of chromatin-bound Mcm2 were not dramatically different, except for a delay in Mcm2 removal from chromatin in the presence of high [dNTP], consistent with a delay in passage through S phase (Fig. 4). The dynamics of new bud appearance was similar in the gal-induced *dbf2-1* pGAL-*RNR1* and *dbf2-1* pGAL-*rnr1-D57N* strains after release from the mitotic arrest [supporting information (SI) Fig. 6].

Induced *pGAL-RNR1* Is Synthetic Sick with *orc2-1* and *orc5-1*. It is known that the number of origins of DNA replication in yeast are in excess of what is normally required to complete S phase (24). Because constitutive, high [dNTP] blocked initiation of DNA replication from the normal number of pre-RCs and slowed cell proliferation, we predicted that lowering the number of pre-RCs might further compromise cell proliferation in the presence of high dNTP. A low number of active origins of DNA replication are present in strains harboring mutations in origin recognition complex subunits, such as *orc2-1* and *orc5-1,* even when grown at a permissive or semipermissive temperature (25).

Moderate [dNTP] that do not block cell cycle progression in wild-type strains were induced in *orc2-1* or *orc5-1* strains containing pGAL-*RNR1*. In both cases a synthetic sickness was observed (Fig. 5 *A* and *B*), suggesting that S-phase progression from a limited number of origins is sensitive to even moderately elevated [dNTP]. Induction of pGAL-*rnr1-D57N* was synthetic lethal with both *orc2-1* (data not shown) and *orc5-1* mutants [Fig. 5*C*; note that at 33°C the

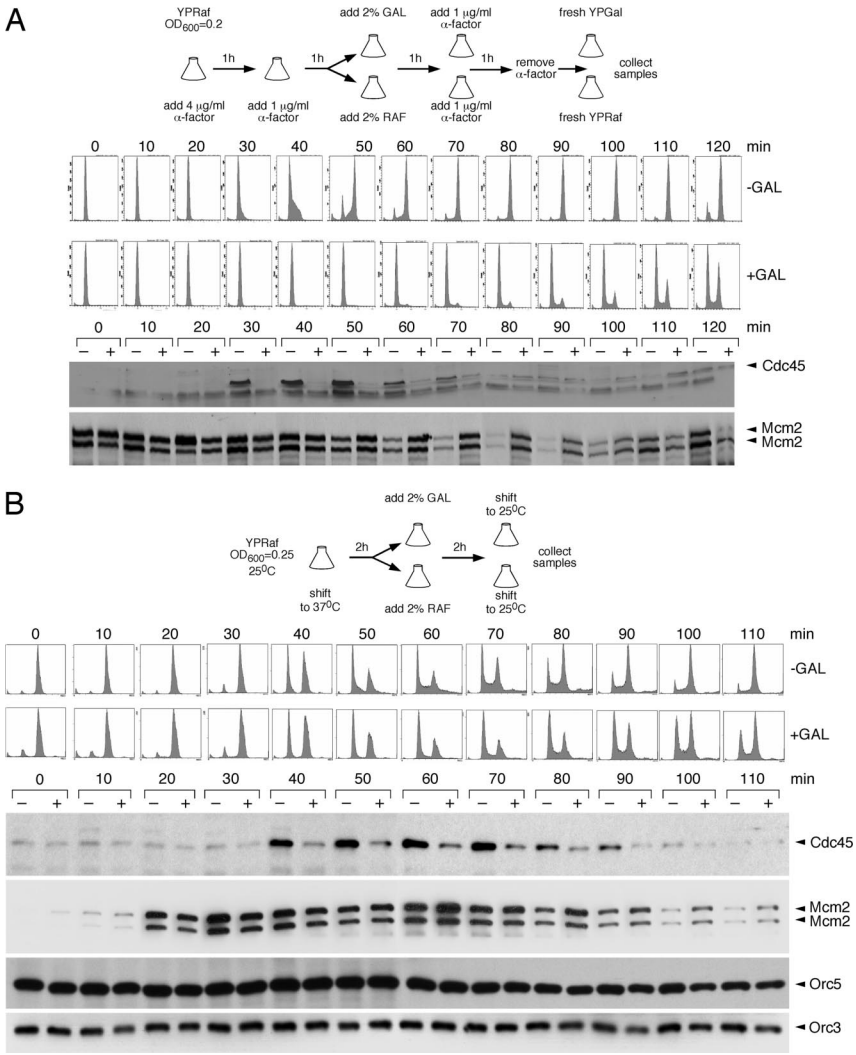

Fig. 4. Loading of Cdc45 onto chromatin is inhibited by expression of pGAL-*rnr1-D57N*. (*A*) pGAL-*rnr1-D57N* (AC439) strain was treated as shown in the outline. The samples were collected at 10-min intervals for analysis of Cdc45 and Mcm2 association with chromatin and for flow-cytometric analysis. (*B*) The pGAL-*rnr1-D57N dbf2-1* (AC473) strain was treated as shown in the outline. The samples were collected at 10-min intervals for analysis of Cdc45, Mcm2, Orc3, and Orc5 association with chromatin and for flow-cytometric analysis.

pGAL-*rnr1-D57N* strain grows slightly faster than at 28–30°C, perhaps because of a lower RNR activity at higher temperatures (26)]. These results further suggest that activation of origins of DNA replication is sensitive to preexisting high [dNTP].

Discussion

To understand the role of [dNTP] fluctuation during the cell cycle, we overexpressed a wild-type and an overactive RNR. Expression of *RNR1* resulted in a 9- to 10-fold increase in [dNTP] and a 1.2- to 1.8-fold decrease in [NTP], but no apparent growth defects. Expression of the overactive *rnr1-D57N* resulted in an ≈35-fold increase in [dNTP], a 1.3- to 2.2-fold decrease in [NTP], retardation of cell proliferation, and defects in the DNA damage checkpoint. Because the difference in [dNTP] between the gal-induced pGAL-*RNR1* and pGAL-*rnr1-D57N* strains was significantly bigger than the difference in [NTP] in these strains and because we found no defects in the rate of rRNA production in the induced pGAL-*rnr1-D57N* strain, we suggest that the proliferation defects observed in the pGAL-*rnr1-D57N* strain are mainly due to the higher [dNTP] and not the lower [NTP].

A transient ≈30-fold increase in [dNTP] in response to DNA damage in the strain with the *rnr1-D57N* allele under its own promoter increases DNA damage tolerance but does not cause cell proliferation defects (17). Thus, it is not the high [dNTP] *per se*, but its continuous presence during the cell cycle that is deleterious to cell proliferation. The flow-cytometric analysis of the pGAL-*rnr1-D57N* strain with high [dNTP] showed that cells accumulate in G_1 phase, where the [dNTP] is usually low. An important process that can occur only in G_1 phase is the assembly of DNA replication origins. We found that high [dNTP] in G_1 phase did not significantly inhibit minichromosome maintenance proteins loading onto chromatin, and hence pre-RC assembly was most likely normal. But activation of pre-RCs was defective based on the slow assembly of Cdc45, a component of the pre-IC, onto chromatin. Even a moderate increase in [dNTP] after the pGAL-*RNR1* induction resulted in synthetic sickness in the origin recognition complex mutants *orc2-1* and *orc5-1*. Although we cannot completely exclude that the observed phenotypes are not due to some unknown activity of the Rnr1 protein, we propose that it is the continuously high [dNTP] present in the induced pGAL-*RNR1* and pGAL-*rnr1-D57N* strains that affects processes regulated by dNTP in G_1 and early S phase.

DNA damage activates RNR in different phases of the cell cycle including G_1 (19). Because high [dNTP] inhibits pre-IC assembly

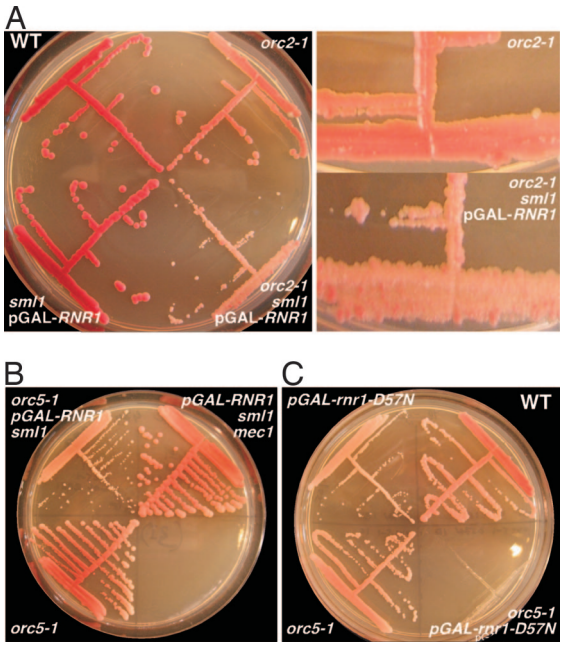

Fig. 5. Elevation of [dNTP] results in synthetic sickness or lethality in the *orc2-1* and *orc5-1* mutants. (*A*) Wild-type (AC476-1D), *orc2-1* (AC476-1A), *orc2-1 sml1* pGAL-*RNR1* (AC476-5B), and *sml1* pGAL-*RNR1* (AC475-4I) strains were streaked on YPGal and incubated at 24°C. (*Right*) A magnification showing synthetic sickness of *orc2-1* plus pGAL-*RNR1* (slower growth, uneven colonies). (*B*) *orc5-1* (AC474-3H), *orc5-1 sml1* pGAL-*RNR1* (AC474-3G), and *sml1 mec1* pGAL-*RNR1* (AC474-3F) strains were streaked on YPGal and incubated at 33°C. (*C*) Wild-type (AC459-1A), *orc5-1* pGAL-*rnr1-D57N* (AC459-1D), *orc5-1*(AC459-1C), and pGAL-*rnr1-D57N* (AC459-1B) strains were streaked on YPGal and incubated at 33°C.

and hence activation of existing pre-RCs, an elevated [dNTP] in G_1 phase, such as occurs after DNA damage, might be one of the effectors that delays entry into S phase. These observations may explain why there are multiple regulatory mechanisms to limit RNR activity in G_1 phase.

It is apparent that the constant, high [dNTP] blocks DNA replication in the absence of the Rad53-dependent checkpoint. One possibility is that the checkpoint may not be induced because of the low number of origins that are activated in S phase. For example, when cells traverse S phase with a suboptimal number of replication forks, they are unable to activate Rad53 and become DNA-damage-sensitive (27, 28). Alternatively, the S-phase DNA damage checkpoint may be inhibited by the constitutive, high [dNTP] directly blocking Rad53 activation, perhaps by feedback inhibition. It could be that, in the presence of constitutive, high [dNTP], the checkpoint cannot get started. Whatever the mechanism, the observation that the DNA damage checkpoint is inactive in the presence of high [dNTP] explains why such cells are sensitive to DNA-damaging reagents. But these results also suggest that dNTPs inhibit initiation of DNA replication by a checkpoint-independent process that regulates pre-IC assembly.

In wild-type strains the [dNTP] present after DNA damage is ≈4-fold above [dNTP] in an unperturbed S phase (17). It is important to note, however, that this dNTP induction is transient. The elevated [dNTP] present after DNA damage in S phase may be one of the direct cellular signals that blocks activation of downstream pre-RCs or helps to maintain these pre-RCs in an inactive state, thereby preventing initiation of DNA replication on damaged templates until the repair is completed. Once [dNTP] returns to normal and the checkpoint is no longer functioning, activation of pre-RCs that have not yet been used in S phase facilitates the completion of DNA replication.

Can dNTP function as regulatory molecules *in vivo*? There are several examples of proteins that have higher affinity to dNTP compared with NTP and that become activated at lower [dNTP] than corresponding [NTP]. A key regulator of apoptosis, Apaf-1, is activated by lower [dATP] than [ATP] *in vitro* and was reported to bind dATP and not ATP *in vivo* (29, 30). Recently, both dATP and ATP were shown to bind cytochrome *c* and prevent its activation of Apaf-1 (31). The activity of RecA, a regulator of DNA recombination in bacteria, is enhanced if dATP is used *in vitro* in place of ATP (32, 33).

What might be the targets for dNTPs blocking pre-IC assembly? It is possible that S-phase cyclin-CDK (Clb5/6-Cdc28) and Cdc7-Dbf4 required for activation of pre-IC are inhibited by dNTP. Cdc7/Dbf4 kinase has been implicated as a target of the Rad53-dependent intra-S-phase checkpoint response by blocking activation of pre-RCs that have not fired, although a Cdc7-independent checkpoint pathway exists (34). This alternative pathway might involve Mcm10 or primase, both of which are required for origin activation and have a nucleotide binding site. Mutations in primase and its associated DNA polymerase α have been implicated in the intra-S-phase checkpoint (35, 36). Finally, because the pre-RC proteins, including origin recognition complex, Cdc6, and minichromosome maintenance proteins, bind nucleotide, high [dNTP] might block their activation in the pre-RC.

Is continuous RNR/dNTP presence deleterious in higher eukaryotes? In mammalian cells, the cell cycle-regulated component of RNR is the small subunit called R2 (1, 37). It is ubiquitylated and degraded by anaphase-promoting complex-Cdh1/proteasome in mitosis and resynthesized in the late G_1 phase after its transcription is derepressed (7, 8, 38). Interestingly, although it is easy to obtain R2-expressing untransformed fibroblasts by transformation with the full R2 gene under its own, cell cycle-regulated promoter, numerous attempts to express R2 protein in untransformed fibroblasts using R2 cDNA under SV40 promoter failed (L. Thelander, personal communication). It has been proposed that constant expression of R2 protein from the SV40 early promoter is lethal to cells, but no mechanism for this lethality has been proposed (39). This observation suggests that also in mammalian cells the unscheduled expression of RNR is incompatible with normal cell proliferation.

In contrast to dATP, dCTP, and dGTP, the concentration of dTTP is regulated not only by RNR, but also by dCMP deaminase and thymidylate kinase (TMPK). This is because there is no rTDP precursor *in vivo* and dTTP is made from dUDP or dCDP first produced by RNR (1). In addition, in mammalian cells the substrate cycle (phosphorylation of thymidine by thymidine kinases and degradation of dTMP by 5′-deoxynucleotidases), together with thymidine phosphorylase, plays an important role in the regulation of [dTTP] (1, 16, 40). Interestingly, human thymidine kinase TK1 and TMPK are also targets for the anaphase-promoting complex/C pathway and, similar to RNR, are degraded in late mitosis (41, 42). Overexpression of the wild-type TK1 and TMPK did not inhibit cell growth, whereas overexpression of the nondegradable TK1 and TMPK led to a severe growth retardation (42). The levels of the wild-type and nondegradable proteins were similar, suggesting that it was the presence of TK1 and TMPK proteins in G_1 phase that led to growth inhibition. The authors attributed slow cell proliferation to the severe imbalance of dNTP pools in the cells expressing nondegradable proteins, but, in light of our results, it is also possible that the presence of high [dTTP] in G_1 phase affected activation of replication origins in mammalian cells.

A recent report has demonstrated an intimate link between dNTP production and the initiation of DNA replication in *Escherichia coli*, but in this case initiation of DNA replication controls the synthesis of RNR subunit transcription, linking activation of the initiation complex to dNTP production (43). In *S. cerevisiae* the link between dNTP production and origin activation may be a two-way interaction. It will be interesting to analyze the effects of increased

[dNTP] on the activation of pre-RC in organisms that do not increase [dNTP] after DNA damage above the S-phase concentration, like *S. pombe* and the mammalian fibroblasts (10, 44). In these cells even a moderate increase of [dNTP] might be affecting the activation of pre-RC.

Materials and Methods

Yeast Strains and Media. Yeasts were grown in YP (1% yeast extract, 2% peptone) with 2% dextrose (YPD), 2% galactose (YPGal), or 2% raffinose (YPRaf). To construct pGAL, pGAL-*RNR1*, and pGAL-*rnr1-D57N* strains, *RNR1* and *rnr1-D57N* were PCR-amplified by using previously described pET21a-*RNR1* and pET3a-*rnr1-D57N* plasmids as templates (17) and cloned in the pESC-URA vector (Stratagene, La Jolla, CA) between BamHI and KpnI restriction sites. To remove the 2μ origin, the vectors were digested with AfeI, purified by electrophoresis, and ligated. The resulting vectors were linearized in the *URA3* gene by digestion with StuI and transformed into W1588-4C. The C-terminally HA-tagged *RNR3* was constructed by a one-step PCR-mediated technique (45). After transformation all stains were confirmed by PCR and back-crossed to W1588-4A. Other strains listed in SI Table 1 were obtained by standard genetic manipulations.

dNTP and NTP Analysis. Isolation of nucleotides from yeast was done as described (17). dNTP and NTP were analyzed by HPLC on a PolyWAX LP column (PolyLC, Columbia, MD) by using a UV-2075 Plus detector (Jasco, Tokyo, Japan). Nucleotides were isocratically eluted with 2.5% acetonitrile/0.3 M potassium phosphate (pH 5.0) buffer.

Flow Cytometry. Samples for flow cytometry were prepared as described in ref. 46 and analyzed on a Cytomics FC500 (Beckman Coulter, Fullerton, CA) or a LSR II (Becton Dickinson).

RNA Isolation and Labeling. RNA isolation and labeling were done as described (47–49). Cells were collected by centrifugation and resuspended in 0.5 ml of buffer (50 mM sodium acetate, 10 mM EDTA, and 0.1% SDS). RNA was extracted with 0.5 ml of phenol preheated to 65°C, followed by phenol-chloroform (1:1) preheated to 65°C. RNA was precipitated by ethanol, fractionated on a 1.5% agarose gel containing 6.7% formaldehyde, and stained by ethidium bromide. For rRNA labeling, the induced and uninduced pGAL-*rnr1-D57N* cultures were adjusted to an OD_{600} of 0.4, and 1.5 ml was incubated with L-[methyl-H^3]-methionine [60 μCi/ml final concentration (1 Ci = 37 GBq)] for 5 min. The gel was stained with ethidium bromide, photographed, treated with EN3HANCE (PerkinElmer, Wellesley, MA), dried, and exposed to film with an enhancer screen at −80°C.

Chromatin Binding Assay. Yeast cells were synchronized by temperature arrest or α-factor arrest as outlined in Fig. 4. The cells were centrifuged, resuspended in 1 ml of CSB buffer (100 mM Pipes·KOH, pH 9.4/10 mM DTT), and incubated at 30°C for 10 min. After 2 min of centrifugation at 2,000 × *g*, the cells were resuspended in 1 ml of SB buffer (50 mM phosphate buffer, pH 7.5/0.6 M sorbitol/10 mM DTT), and, after addition of 40 μl of zymolyase (2 mg/ml in 1 M sorbitol), incubated for 15 min at 30°C. The spheroplasts were centrifuged for 2 min at 2,000 × *g*, resuspended in 1 ml of ice-cold SWB buffer [50 mM Hepes·KOH, pH 7.5/2.5 mM $MgCl_2$/0.4 M sorbitol/protease inhibitors (Roche, Basel, Switzerland)], and centrifuged for 2 min at 2,000 × *g*. The pellets were resuspended in 40 μl of ice-cold EB buffer [100 mM KCl/50 mM Hepes·KOH, pH 7.5/2.5 mM $MgCl_2$/0.25% Nonidet P-40/protease inhibitors (Roche)] and underlaid with 20 μl of 30% sucrose. After 10 min of centrifugation at 10,000 × *g*, pellets were washed with EB buffer, resuspended in 40 μl of EB buffer, mixed with 40 μl of SDS/PAGE loading buffer, and boiled.

Western Blotting. For Rad53 detection, yeast cells were lysed as described (50) and analyzed with an anti-Rad53 FHA1(20–164) rabbit polyclonal antibody (1:5,000). For Rnr3-HA detection, protein extract was prepared as described (51) and used with anti-HA-tag 12CA5 mouse monoclonal antibody (1:5,000). For analysis of the chromatin-bound Cdc45, Mcm2, Orc3, and Orc5, chromatin fractions were probed with anti-Cdc45 CS1485 rabbit polyclonal antibody (0.5 μg/ml), anti-Orc3 SB3 mouse monoclonal antibody (1:2,000), anti-Mcm2 mcm2–39 mouse monoclonal antibody (1:2,000), and anti-Orc5 SB5 mouse monoclonal antibody (1:4,000).

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1. Reichard P (1988) *Annu Rev Biochem* 57:349–374.
2. Elledge SJ, Zhou Z, Allen JB (1992) *Trends Biochem Sci* 17:119–123.
3. Nordlund P, Reichard P (2006) *Annu Rev Biochem* 75:681–706.
4. Mathews CK (2006) *FASEB J* 20:1300–1314.
5. Thelander L, Reichard P (1979) *Annu Rev Biochem* 48:133–158.
6. Elledge SJ, Zhou Z, Allen JB, Navas TA (1993) *BioEssays* 15:333–339.
7. Chabes AL, Pfleger CM, Kirschner MW, Thelander L (2003) *Proc Natl Acad Sci USA* 100:3925–3929.
8. Chabes AL, Bjorklund S, Thelander L (2004) *J Biol Chem* 279:10796–10807.
9. Liu C, Powell KA, Mundt K, Wu L, Carr AM, Caspari T (2003) *Genes Dev* 17:1130–1140.
10. Hakansson P, Dahl L, Chilkova O, Domkin V, Thelander L (2006) *J Biol Chem* 281:1778–1783.
11. Zhao X, Muller EG, Rothstein R (1998) *Mol Cell* 2:329–340.
12. Zhao X, Chabes A, Domkin V, Thelander L, Rothstein R (2001) *EMBO J* 20:3544–3553.
13. Yao R, Zhang Z, An X, Bucci B, Perlstein DL, Stubbe J, Huang M (2003) *Proc Natl Acad Sci USA* 100:6628–6633.
14. Lee YD, Elledge SJ (2006) *Genes Dev* 20:334–344.
15. An X, Zhang Z, Yang K, Huang M (2006) *Genetics* 173:63–73.
16. Pontarin G, Ferraro P, Valentino ML, Hirano M, Reichard P, Bianchi V (2006) *J Biol Chem* 281:22720–22728.
17. Chabes A, Georgieva B, Domkin V, Zhao X, Rothstein R, Thelander L (2003) *Cell* 112:391–401.
18. Huang M, Zhou Z, Elledge SJ (1998) *Cell* 94:595–605.
19. Elledge SJ, Davis RW (1990) *Genes Dev* 4:740–751.
20. Santocanale C, Diffley JF (1998) *Nature* 395:615–618.
21. Sun Z, Fay DS, Marini F, Foiani M, Stern DF (1996) *Genes Dev* 10:395–406.
22. Sanchez Y, Desany BA, Jones WJ, Liu Q, Wang B, Elledge SJ (1996) *Science* 271:357–360.
23. Zou L, Stillman B (1998) *Science* 280:593–596.
24. Dutta A, Bell SP (1997) *Annu Rev Cell Dev Biol* 13:293–332.
25. Liang C, Weinreich M, Stillman B (1995) *Cell* 81:667–676.
26. Chabes A, Domkin V, Larsson G, Liu A, Graslund A, Wijmenga S, Thelander L (2000) *Proc Natl Acad Sci USA* 97:2474–2479.
27. Tercero JA, Longhese MP, Diffley JF (2003) *Mol Cell* 11:1323–1336.
28. Shimada K, Pasero P, Gasser SM (2002) *Genes Dev* 16:3236–3252.
29. Kim HE, Du F, Fang M, Wang X (2005) *Proc Natl Acad Sci USA* 102:17545–17550.
30. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) *Cell* 91:479–489.
31. Chandra D, Bratton SB, Person MD, Tian Y, Martin AG, Ayres M, Fearnhead HO, Gandhi V, Tang DG (2006) *Cell* 125:1333–1346.
32. Menetski JP, Kowalczykowski SC (1989) *Biochemistry* 28:5871–5881.
33. Shan Q, Bork JM, Webb BL, Inman RB, Cox MM (1997) *J Mol Biol* 265:519–540.
34. Jares P, Donaldson A, Blow JJ (2000) *EMBO Rep* 1:319–322.
35. Tan S, Wang TS (2000) *Mol Cell Biol* 20:7853–7866.
36. Longhese MP, Foiani M, Muzi-Falconi M, Lucchini G, Plevani P (1998) *EMBO J* 17:5525–5528.
37. Eriksson S, Martin DW, Jr (1981) *J Biol Chem* 256:9436–9440.
38. Bjorklund S, Skog S, Tribukait B, Thelander L (1990) *Biochemistry* 29:5452–5458.
39. Thelander L, Berg P (1986) *Mol Cell Biol* 6:3433–3442.
40. Ferraro P, Pontarin G, Crocco L, Fabris S, Reichard P, Bianchi V (2005) *J Biol Chem* 280:24472–24480.
41. Ke PY, Chang ZF (2004) *Mol Cell Biol* 24:514–526.
42. Ke PY, Kuo YY, Hu CM, Chang ZF (2005) *Genes Dev* 19:1920–1933.
43. Gon S, Camara JE, Klungsoyr HK, Crooke E, Skarstad K, Beckwith J (2006) *EMBO J* 25:1137–1147.
44. Hakansson P, Hofer A, Thelander L (2006) *J Biol Chem* 281:7834–7841.
45. Longtine MS, McKenzie A, III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998) *Yeast* 14:953–961.
46. Haase SB, Reed SI (2002) *Cell Cycle* 1:132–136.
47. Powers T, Walter P (1999) *Mol Biol Cell* 10:987–1000.
48. Warner JR (1991) *Methods Enzymol* 194:423–428.
49. Kohrer K, Domdey H (1991) *Methods Enzymol* 194:398–405.
50. Pike BL, Hammet A, Heierhorst J (2001) *J Biol Chem* 276:14019–14026.
51. Burke D, Dawson D, Stearns T (2000) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).