Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools

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The relationship between dNTP levels and DNA synthesis was investigated using α factor-synchronized yeast treated with the ribonucleotide reductase inhibitor hydroxyurea (HU). Although HU blocked DNA synthesis and prevented the dNTP pool expansion that normally occurs at G1/S, it did not exhaust the levels of any of the four dNTPs, which dropped to about 80% of G1 levels. When dbf4 yeast that are ts for replication initiation were allowed to pre-accumulate dNTPs at 37°C before being released to 25°C in the presence of HU, they synthesized 0.3 genome-equivalents of DNA and then arrested as dNTPs approached sub-G1 levels. Accumulation of dNTPs at G1/S was not a prerequisite for replication initiation, as dbf4 cells incubated in HU at 25°C were able to replicate when subsequently switched to 37°C in the absence of HU. The replication arrest mechanism was not dependent on the Mec1/Rad53 pathway, as checkpoint-deficient rad53 cells also failed to exhaust basal dNTPs when incubated in HU. The persistence of basal dNTP levels in HU-arrested cells, and partial bypass of the arrest in cells that had pre-accumulated dNTPs suggest that cells have a mechanism for arresting DNA chain elongation when dNTP levels are not maintained above a critical threshold.
INTRODUCTION

Hydroxyurea (HU) is a potent inhibitor of the enzyme ribonucleotide reductase (RNR) and inhibits DNA replication in a wide variety of cells, including Saccharomyces cerevisiae (1). The simplest explanation for HU inhibition of DNA synthesis is that it starves the DNA polymerase at the replication forks for dNTPs. HU treatment has been shown to reduce the purine dNTP pools in a variety of mammalian cells (2-7); however, conflicting data exist concerning its modulation of pyrimidine dNTP pool levels. Furthermore, even for purine dNTPs, HU has only rarely been shown to cause a complete depletion of the dGTP or dATP pools (2-4). More commonly, HU results in only partial depletion of the purine dNTP pools (5-7). The complicated, often reciprocal, changes in individual dNTP pools that occur in HU-treated mammalian cells may be due to the compensatory activities of deoxyribonucleotide salvage pathways in higher eukaryotes. Budding yeast offers a simpler system in which to study the mechanism by which HU affects replication. Yeast possess no deoxyribonucleoside kinase activities and thus deoxyribonucleotide synthesis is entirely dependent on ribonucleotide reductase. Also, yeast can easily be synchronized in G1 using mating pheromone, and the availability of several temperature-sensitive cdc mutations allows cell cycle progression to be reversibly halted at specific points throughout the cell cycle (8). Reciprocal switch experiments in yeast have ordered the execution point of several cdc genes with respect to the HU-sensitive step during the cell cycle (9). Furthermore, mutational screens in yeast have identified regulatory proteins, such as Mec1 and Rad53, that are necessary for proper execution of the HU-induced replication arrest checkpoint (10). Despite the genetic advantages of S. cerevisiae, the relative simplicity of its dNTP synthesis pathway, and the well studied transcriptional control of its dNTP-synthesizing genes (11-14), relatively few analyses of dNTP levels in yeast have been done. Three studies have reported dNTP measurements in asynchronously growing yeast (15-17). We and others
(18,19) recently showed that dNTP levels oscillate during the yeast cell cycle, with peak levels occurring shortly after the G1/S transition. Zhao et al. (16) showed that deletion of Sml1, a Mec1/Rad53-regulated protein that binds and inhibits RNR, results in dNTP pool expansion. Chabes et al. (19) showed that RNR1 gene mutations that free RNR enzyme from allosteric feedback inhibition result in elevated dNTP levels and increased resistance to DNA-damaging agents. There is no published information on the effects of HU on dNTP levels in yeast.

In mammalian cells, the mechanism by which HU inhibits replication has been investigated by attempting to reverse HU inhibition by administration of exogenous deoxyribonucleosides (20-22). In yeast, the absence of deoxyribonucleotide salvage enzymes makes it difficult to assay the ability of exogenous deoxyribonucleosides to reverse the effects of HU. However, the availability of cdc mutants that cannot initiate S phase at the non-permissive temperature represents a potential means of genetically elevating dNTP levels prior to application of HU. The Dbf4 polypeptide, together with Cdc7, form a heterodimeric protein kinase that is essential for replication initiation (23). The Dbf4/Cdc7-requiring process is the last genetically identified step in the pathway linking START to replication origin firing (23). We used a mutant dbf4 strain to investigate whether pre-accumulation of dNTPs bypasses the inhibitory effect of HU on replication.

The dbf4 strain was used to test another aspect of the relationship between dNTP accumulation and replication initiation. In yeast (18,19) and several mammalian cell lines (24-26), experiments have shown that dNTPs accumulate as synchronized cells enter S phase. The concomitant accumulation of dNTPs and synthesis of DNA suggests that there might be a link between the initiation of these two events. In the current study, we tested whether a threshold level of dNTPs was needed for replication initiation by releasing α factor-arrested dbf4 yeast into HU medium at the permissive temperature and then monitoring their ability to replicate when switched to the non-permissive
temperature in the absence of HU. A similar reciprocal switch experiment in which DNA synthesis and cell division were monitored has already established that Cdc7, the protein with which Dbf4 interacts, acts prior to the HU-sensitive step during the cell cycle (9). This suggests that dNTP accumulation is not required for replication initiation. However, in this and all other previous reciprocal switch experiments involving HU and cdc mutations, dNTP levels were never measured. Furthermore, although Cdc7 has been analyzed, that Dbf4 acts prior to HU in preventing replication has not formally been shown.

The checkpoint kinases Mec1 and Rad53 act sequentially in a pathway that, among other things, appear to boost or preserve dNTP levels in cells treated with DNA-damaging agents or HU. In response to DNA damage, the Mec1/Rad53 pathway positively regulates RNR activity in at least three ways. It stimulates transcription of the large and small subunit $RNR$ genes (27). It is needed for the redistribution of the RNR small subunit (Rnr2) from the nucleus to the cytoplasm, where it can join with the large subunit (Rnr1) to form the holoenzyme (28). It inactivates the RNR inhibitor Sml1 (16,29,30). The Mec1/Rad53 pathway also negatively regulates replication origin activity in HU-treated cells. In wild-type yeast, HU blocks DNA chain initiation at late replication origins (27,31). In $rad53$ mutants, HU does not block the firing of late origins (31,32). The occurrence of DNA synthesis at late origins in $rad53$ cells suggested that HU-treated cells must have a residual pool of dNTPs. To determine the extent to which dNTP pools fall in HU-treated cells, the levels of all four dNTPs were determined in synchronized wild-type and $rad53$ cells incubated in the absence and presence of HU.

Our analyses showed that, although HU prevented dNTP accumulation at G1/S and blocked DNA synthesis, it did not exhaust the basal dNTP pools. HU inhibition of replication was bypassed if $dbf4$ cells were allowed to pre-accumulate dNTPs at the non-permissive temperature prior to exposure to the drug at the permissive temperature. Accumulation of dNTPs was not a prerequisite for replication initiation, as $dbf4$ cells
released from α factor into HU at the permissive temperature were able to replicate when switched to the non-permissive temperature in the absence of HU. Finally, the arrest mechanism was not dependent on the Mec1/Rad53 pathway, as HU treatment did not exhaust the dNTP pools in checkpoint-deficient rad53 mutants. Our results suggest that cells have evolved a mechanism for arresting DNA chain elongation when dNTP fall below a minimum threshold. Elongation arrest may represent a means of conserving basal dNTPs for processes such as DNA repair.
EXPERIMENTAL PROCEDURES

Media, Strains and General Methods

Cells were grown in YEPD medium. Yeast strains used are listed in Table 1. MY273 and SY2626 are derivatives of W303-1 that carry a bar1 mutation to facilitate α factor synchronization. To derive MY273, W303-1α was mated to SY2626 (G. Sprague, U. Oregon, Eugene, OR) and a mat-α segregant carrying the bar1 allele was isolated by tetrad dissection. To derive the dbf4-1 bar1 strain MY317, MY273 was mated to L128-20 (L. Johnston, National Institute for Medical Research, Mill Hill, London) and random spores from the resulting diploid were screened for α factor and temperature sensitivity. To derive the rad53-11 bar1 strain MY376, MY273 was mated to BS883 (R. Sclafani, U. of Colorado Medical Center, Denver, CO) and tetrads from the resulting diploid were dissected and tested for α factor sensitivity and uracil prototrophy. (The rad53-11 mutation was linked to pep4:URA3.) The wild-type RAD53 strain (MY377) was derived from the same tetrad used to derive MY376.

G1-arrested cells were obtained by incubating asynchronously-growing cells with 100 ng/ml α factor for 3 hours at 25°C. To achieve synchrony, arrested cells were collected on Whatman #1 filters, washed once with fresh YEPD and once with 100% conditioned medium, resuspended in 25% conditioned medium, and incubated at either 25°C or 37°C. Conditioned medium was prepared by growing W303-1a cells to saturation in YEPD and removing the cells by filtration.

Flow cytometry

About 5 x 10^6 cells were collected by centrifugation and fixed in 70% ethanol overnight. After microcentrifugation (10,000 rpm, 5 sec), the cell pellet was washed with 100 µl 50 mM sodium citrate buffer (pH 7), resuspended in 100 µl sodium citrate buffer and sonicated for 6 seconds. After a 1-hr incubation at 50°C with RNase-A (0.25
mg/ml) and a 1-hr incubation with proteinase K (1 mg/ml), 100 µl of sodium citrate buffer containing 16 µg/ml propidium iodide was added and samples were incubated at 4°C overnight in the dark. A Beckman Coulter Epics XL flow cytometer was used to analyze the samples. For each histogram, 25,000 cells were assayed.

**dNTP pool measurements**

Approximately 3 x 10^8 cells were harvested and extracted as described by Muller (15). Each precipitated sample was resuspended in 200 µl cold H₂O and assayed for each of the four dNTPs by the DNA polymerase-based enzymatic assay (33), which is based on the incorporation of a limiting dNTP into an alternating co-polymer template poly(dA-dT) or poly(dI-dC) by Klenow DNA polymerase in the presence of an excess of ^3H-labeled complementary dNTP.
RESULTS

HU prevents dNTP accumulation at G1/S in \( \alpha \) factor-synchronized yeast

Wild-type cells (MY377) were synchronized by \( \alpha \) factor arrest and release. As shown by the flow cytometry data in Fig. 1A, asynchronously growing cells arrested with a G1 DNA content when incubated with \( \alpha \) factor, and arrested cells entered S phase about 40 minutes after \( \alpha \) factor was removed. Parallel dNTP pool measurements (Fig. 1B) showed that the levels of all four dNTPs were lower in G1-arrested cells than in asynchronous cells, and that the levels of all four dNTPs increased 2.5- to 4-fold as cells entered S phase. The relative size of each dNTP pool differed reproducibly, with dTTP always being the largest and dGTP always being the smallest. For example, \( 10^8 \) asynchronous cells contained 293 pmol dTTP, 183 pmol dATP, 74 pmol dCTP and 65 pmol dGTP. Assuming a cell volume of 42 femtoliters (34), the concentrations of dTTP, dATP, dCTP and dGTP were 70 \( \mu \)M, 44 \( \mu \)M, 18 \( \mu \)M and 15 \( \mu \)M, respectively.

HU has been shown to alter the dNTP pools of mammalian cells through its interaction with ribonucleotide reductase (2-5). In general, the purine dNTP pools are the most strongly affected by HU; however, in experiments using different mammalian cells, the effects of HU on individual dNTP pools varied quantitatively and qualitatively. Yeast represented a system where cell synchrony could be easily achieved, and where the effects of HU on individual dNTP pools as cells entered S phase could be determined. In particular, we were interested in determining whether any dNTP pool was exhausted as cells began replicating.

As shown by the flow cytometry data in Fig. 1C, when \( \alpha \) factor-arrested MY377 cells were released to fresh medium containing HU, DNA content did not increase, confirming that HU blocked replication. As shown in Fig. 1D, HU prevented the accumulation of dNTPs that normally began about 40 min after \( \alpha \) factor release in the absence of HU (see Fig. 1B). The result suggested that HU blocked the synthesis of all
four dNTPs in yeast. HU affected the dNTP pools evenly, and did not significantly change the ratios of the pools relative to each other. For example, the dTTP : dATP : dCTP : dGTP ratio was 39 : 19 : 11 : 10 before release into HU medium, and was 45 : 20 : 13 : 10 after one hour in HU. After 80 min in HU, the pools had decreased 21% for dTTP, 18% for dATP, 29% for dCTP and 15% for dGTP. On average, dNTP pools contracted to about 79% of G1-arrested levels. Thus, although HU completely blocked DNA replication, it had only a modest effect on dNTP levels.

**Pre-accumulation of dNTPs permits replication in the presence of HU**

The persistence of significant dNTP pools in HU-treated yeast belied the simple idea that replication arrest in HU was due to exhaustion of the substrates needed for chain elongation by DNA polymerase. Careful examination of the results of earlier studies in mammalian cells showed similar persistence of dNTPs and suggested that HU can inhibit replication without exhausting a bulk dNTP pool. Several alternative explanations for HU-induced replication arrest are conceivable. First, cells may have a mechanism for sensing the size of the dNTP pools and halting replication fork progression when a dNTP pool falls to a low, but non-zero, level. Second, inhibition of RNR may completely exhaust a privileged dNTP pool at replication forks and yet only mildly affect the bulk dNTP pools measured in whole cells. Third, inhibition of flux through RNR may inhibit replication by a dNTP-independent mechanism. For example, inhibition of RNR activity may affect the redox state of the co-substrate thioredoxin, and thereby affect the activity of regulatory proteins that are sensitive to the redox state of thioredoxin. Fourth, although RNR is the only characterized target of HU, the drug may affect the activity of an, as yet, uncharacterized target that is required for replication fork progression. The latter three of these explanations predict that HU should continue to block replication if a means of artificially raising the bulk dNTP pools were achieved. The first of these explanations predicts that cells should be able to bypass the HU-induced replication
block if a means of raising the bulk dNTP pools were achieved.

As a means of raising the bulk dNTP pools in yeast we investigated the use of a temperature-sensitive \textit{dbf4} mutation. Dbf4, together with Cdc7, form a holoenzyme with protein kinase activity. The Dbf4/Cdc7 protein kinase is located at replication origins and catalyzes the last known step in the cascade of events linking START to replication origin firing (23). At the non-permissive temperature (37°C), \textit{dbf4} mutants arrest at G1/S with unfired origins of replication.

\textbf{Fig. 2} shows the results obtained when \alpha{} factor-arrested \textit{dbf4} mutants were released from pheromone at the non-permissive temperature in the absence of HU and were subsequently switched to the permissive temperature in the presence of HU.

Previous continuous labeling studies, in which \[^{14}\text{C}]\text{uracil} incorporation into DNA was monitored, indicated that HU inhibits DNA synthesis in yeast within 10 minutes of its administration (9). To insure that HU had adequate time to enter the cell and inactivate RNR, the drug was added 20 minutes before the temperature shift-down. \textbf{Fig. 2B} shows that all four dNTP pools decreased when asynchronous \textit{dbf4} cells (black bars) were arrested in G1 with \alpha{} factor, and that all four dNTP pools increased 2.4- to 4.4-fold when \alpha{} factor was removed and cells were incubated at the non-permissive temperature (37°C) for 80 min (shaded bars, labeled 20, 40, 60 and 60 + 80). Expansion of the dNTP pools at the non-permissive temperature implied that the mechanism responsible for increased dNTP synthesis in late G1 was not dependent on Dbf4. Having pre-accumulated dNTPs at 37°C, would \textit{dbf4} cells switched to the permissive temperature be able to synthesize DNA in the presence of HU? The decrease in dNTP levels that was observed when cells were given HU at 60 min and switched to 25°C at 80 min (\textbf{Fig. 2B}, open bars labeled 60 + 20, 60 + 40, 60 + 60, 60 + 80 and 60 + 100) was consistent with the idea that DNA synthesis was occurring and that dNTPs were being consumed through their incorporation into DNA.

Flow cytometry was used to directly monitor DNA synthesis in cells released
from a \textit{dbf4} block in the presence of HU. As shown in \textbf{Fig. 2A}, \textit{dbf4} cells arrested with a G1 DNA content when incubated in \(\alpha\) factor, and did not enter S phase for at least 60 min when \(\alpha\) factor was removed and cells were incubated at the non-permissive temperature (37°C). However, when cells were given HU at 60 min and switched to the permissive temperature (25°C) at 80 min (60 + 20), they began to synthesize DNA. By 20 minutes after the temperature shift-down (60 + 40), DNA content had increased 27% above G1 levels. By this time, dNTP pools had fallen to nearly G1 levels (see \textbf{Fig. 2B}). By 40 minutes after the temperature shift-down (60 + 60), DNA content had increased by 30%, and dNTP pools had fallen to G1 levels. With longer incubations, out to 80 min after the temperature shift-down (60 + 100), DNA content had not increased further, and dNTP pools in general had fallen to a constant, non-zero level. The data in \textbf{Fig. 2A} and \textbf{2B} indicate that when cells were allowed to pre-accumulate dNTPs before HU was added, they were able to replicate about 30% of their genome in the presence of the drug. Then, once dNTPs had dropped to about G1 levels, DNA synthesis once again became arrested.

In the experiment shown in \textbf{Fig. 2}, it was important that HU be added several minutes prior to the temperature shift-down. If HU was added simultaneously with the temperature shift-down, many cells seemed to replicate almost their entire genome. We speculate that when HU is added at the same time as the temperature shift-down, significant amounts of DNA were synthesized before HU had entered the cell and inhibited RNR activity.

From the results in \textbf{Fig. 2}, we concluded that pre-accumulation of dNTPs allowed cells to replicate 30% of their genome in the presence of HU. The result was inconsistent with models in which the HU-induced replication arrest was due to an effect of HU on a privileged dNTP pool, on the redox state of RNR co-substrates such as thioredoxin, or on an as yet uncharacterized target of the drug. The results support a model in which DNA replication in yeast requires a critical dNTP threshold, below which replication forks are arrested. According to this model, HU blocks replication because, in the absence of RNR
activity, cells cannot maintain their dNTP pools above the threshold required to prevent replication arrest.

**Deoxyribonucleotide accumulation is not required for Dbf4 activity**

Although the data in Fig. 2 showed that Dbf4 activity was not required for dNTP accumulation at G1/S, the reciprocal question of whether dNTP accumulation was required for Dbf4 activity at G1/S has not formally been addressed. To investigate this question, dNTP levels and DNA content were measured in α factor-synchronized dbf4 cells released from pheromone at the permissive temperature in the presence of HU and subsequently switched to the non-permissive temperature in the absence of HU. To insure that Dbf4 protein was inactivated prior to restoration of RNR activity, cells were switched to the non-permissive temperature 10 min before HU was removed. As shown in Fig. 3B, dbf4 cells released from pheromone at the permissive temperature in the presence of HU did not accumulate dNTPs (open bars, labeled 20, 40 and 60). When subsequently switched to the non-permissive temperature in the absence of HU, the cells gradually accumulated dNTPs over the next 60 min (shaded bars, labeled 60 + 20, 60 + 40 and 60 + 60). Parallel flow cytometric determination of DNA content (Fig. 3A) showed that cells remained with a G1 DNA content during the initial 60 min incubation at the permissive temperature in the presence of HU, and that most cells acquired a G2 DNA content during a subsequent 60 min incubation at the non-permissive temperature in the absence of HU. The increase in DNA content at the non-permissive temperature shows that the majority of cells were able to traverse the Dbf4 execution point during the initial incubation at the permissive temperature, even though HU was present and prevented dNTP accumulation. As the execution point for the Dbf4/Cdc7 kinase lies very close to the act of replication origin firing, it is likely that origins were able to initiate replication in the absence of dNTP accumulation.
Persistence of dNTP pools in HU-arrested rad53 cells

The persistence of dNTP pools in HU-arrested wild-type cells (see Fig. 1), and bypass of the HU arrest in dbf4 cells that had pre-accumulated dNTPs prior to HU exposure (see Fig. 2), suggested that cells have a mechanism for arresting DNA chain elongation when dNTP levels fall below a minimum threshold. One protein known to play a role in arresting cell cycle progression in HU-treated cells is the protein kinase Rad53 (10). Checkpoint-deficient rad53 cells incubated in HU enter mitosis with unreplicated DNA and die. It was recently shown that checkpoint-deficient rad53 cells are able to synthesize DNA from late-firing origins in the presence of HU (31). As replication from late-firing origins would require dNTPs, we speculated that the mechanism responsible for replication arrest at low dNTP levels was defective in rad53 cells. In other words, DNA replication in HU-treated rad53 may continue until one of the dNTP pools is totally exhausted. To investigate this possibility, DNA content and dNTP levels were determined in α factor-synchronized rad53 cells incubated in the presence or absence of HU.

As shown by the flow cytometry data in Fig. 4A, rad53 cells arrested with a G1 DNA content when incubated in α factor, and began to synthesize DNA within 40 min of α factor removal. Like wild-type cells, when rad53 cells were released from pheromone into medium containing HU (Fig. 4C), most cells retained a G1 DNA content throughout the course of the experiment. Fig. 4B shows dNTP levels in α factor-synchronized rad53 cells. Asynchronous cell dTTP and dATP levels in rad53 cells were similar to wild-type levels, and dCTP and dGTP levels were about 1.7-fold higher than wild-type levels (compare solid bars in Fig. 4B with those in Fig. 1B). Higher asynchronous cell dCTP and dGTP levels in rad53 mutants was somewhat surprising, as the Mec1/Rad53 pathway is known to positively influence RNR activity, at least in DNA-damaged cells (16). As was previously observed for wild-type cells, the levels of all four dNTPs fell when asynchronous rad53 cells were arrested in G1 by incubation in α factor (Fig. 4B, shaded
bar, time zero). Also as previously observed for wild-type cells, dNTP levels increased when rad53 cells were released from α factor. However, the size of the increase was generally smaller in rad53 cells than in wild-type cells. For example, by 60 min after pheromone release, dTTP, dATP, dCTP and dGTP increased 4.0-, 2.1-, 4.0- and 2.1-fold, respectively, in wild-type cells (see Fig. 1B), but increased only 1.6-, 3.1-, 1.2- and 1.3-fold, respectively, in rad53 cells. On average, dNTP levels increased 310% when wild-type cells entered S phase, and increased only 80% when rad53 cells entered S phase. Reduced accumulation of dNTPs in rad53 cells was also evident when maximal absolute levels were compared. Maximal levels of dTTP, dATP, dCTP and dGTP in rad53 cells were 46%, 118%, 49% and 66% that of wild-type cells, respectively. The reduced ability of rad53 cells to accumulate dNTPs as cells entered S phase supports the hypothesis that Rad53 is part of a pathway that positively regulates RNR activity (16).

Fig. 4D shows dNTP levels in α factor-synchronized rad53 cells released to medium containing HU. As was previously observed for wild-type cells, HU blocked dNTP accumulation but did not result in exhaustion of any one of the four dNTP pools. Thus, at least qualitatively, the rad53 mutation did not eliminate the mechanism by which cells arrest replication prior to dNTP exhaustion. After 80 min in HU, the pools had decreased by 19% for dTTP, 25% for dATP, 64% for dCTP and 18% for dGTP. On average, the dNTP pools contracted to about 69% of G1 levels in HU-treated rad53 cells. In comparison, the dNTP pools contracted to about 79% of G1 levels in HU-treated wild-type cells (see Fig. 1D). The 64% decrease in dCTP observed in HU-arrested rad53 cells was noticeably greater than the 29% decrease observed in similarly treated wild-type cells. This inequality was primarily a function of the relatively high levels of dCTP in G1-arrested rad53 cells. If the absolute levels to which dCTP fell were compared, the dCTP pool in HU-arrested rad53 cells was only 18% smaller than in HU-arrested wild-type cells. The rad53 mutation had a similarly small effect on the absolute level to which the other dNTP pools fell in the presence of HU. The dATP pool in HU-
arrested *rad53* cells was 27% smaller than in HU-arrested wild-type cells, and the dTTP and dGTP pools in HU-arrested *rad53* cells were 19% and 27% larger, respectively, than in HU-arrested wild-type cells. Thus, based on either the percent decrease from G1 levels, or the absolute size of the residual dNTP pool, the *rad53* mutation had little effect on the extent to which dNTPs were consumed following HU inhibition of RNR activity. The persistence of dNTP pools in HU-treated *rad53* cells indicated that the mechanism responsible for elongation arrest when dNTP levels are low was not dependent on the Mec1/Rad53 pathway.
DISCUSSION

Most studies on HU-induced replication arrest in S. cerevisiae have focused on the triggering of checkpoint mechanisms that prohibit inappropriate cell cycle transitions and thereby preserve genome stability or prevent catastrophic mitoses (reviewed by Elledge, (10)). In contrast, our work has focused on the most proximal effect of HU – the inhibition of RNR, and the effect of RNR inhibition on dNTP levels and DNA synthesis. Although HU is widely believed to block replication by exhausting the supply of dNTPs required by DNA polymerase, the effect of HU on dNTP levels in yeast had not previously been determined, and published work on the effects of HU on dNTP levels in mammalian cells have been difficult to interpret, perhaps due to the compensatory activities of deoxyribonucleotide salvage pathways. In α factor-synchronized yeast, we found that HU prevented the accumulation of dNTPs that normally occurs as cells enter S phase. Flow cytometry confirmed that HU blocked DNA replication. Although HU blocked dNTP accumulation, it did not completely deplete any one of the four dNTP pools. Our results thus indicated that DNA chain elongation in HU-treated yeast is arrested by a mechanism that preserves basal dNTP pools. Similar preservation of dNTP pools may also occur in HU-treated mammalian cells. For example, when HU is added to exponentially growing human diploid fibroblasts, dATP and dGTP levels decrease only by about 70%, and dCTP and dTTP levels actually increase about 30% (6). Similarly, in mitogen-induced human lymphocytes, although HU prevents the dATP pool expansion that normally accompanies activation, it only lowers dATP levels to about 50% of that present in uninduced cells, and it has even less effect on the three other dNTP pools (5). Also, in chronic myelogenous leukemia, infusion of HU inhibits DNA synthesis but causes only a 60% decrease in dATP and has no effect on dGTP, dCTP or dTTP levels (7). In general, HU has stronger effects on purine deoxyribonucleotides, but even for purines, only rarely has HU been shown to cause complete exhaustion of a
dNTP pool. In serum-synchronized primary mouse embryo cells (2) and in isoleucine-
synchronized CHO cells (3), HU inhibition of DNA synthesis (as measured by
$[^3]H$)thymidine labeling) was associated with strong inhibition of the dGTP pool (2). In mouse
L929 cells synchronized by sub-culturing, HU completely depleted the dATP and dGTP
pools (4). In those cases where HU had a particularly strong effect on dGTP levels, the
dGTP pool was at least an order of magnitude smaller than any other dNTP pool (2,3). In
the above studies, strong effects of HU on purine dNTP levels were observed only in
synchronized cells. In the same studies, HU had relatively mild effects on dNTP levels in
asynchronous cells. For example, in asynchronous L929 cells, HU had little effect on the
size of any dNTP pool, and yet the drug strongly inhibited DNA synthesis (4). Similarly,
in asynchronous mouse embryo cells, HU inhibition of DNA synthesis was not associated
with complete depletion of the dGTP pool (2). Furthermore, in isoleucine-synchronized
CHO cells, although HU blocked the increase in dGTP levels that normally occurs as
cells enter S phase, it had a relatively mild effect on basal dGTP levels, which dropped to
about 70% of G1 levels. In this respect, the CHO cell data were similar to our yeast
results, which showed that HU prevented dNTP accumulation as cells entered S phase,
but did not have much of an effect on basal dNTP levels. The existence of compensatory
salvage pathways in mammalian cells may have contributed to the complicated effects of
HU on individual dNTP levels. Nevertheless, the persistence of dNTPs in most cases
where mammalian cells were treated with HU is consistent with our conclusion that
replication arrest in HU-treated cells is not due to exhaustion of the activated substrates
required by replicative DNA polymerases.

Although our data indicate that HU arrests replication without completely
depleting any dNTP pool, the replication arrest mechanism is not independent of dNTP
levels. If dNTP levels were artificially raised by incubating yeast $dbf4$ mutants at the
non-permissive temperature for S phase entry, HU did not block replication when the
cells were subsequently switched to the permissive temperature (Fig. 2). These results
are consistent with earlier mammalian cell studies, which showed that exogenous deoxyribonucleosides partially or totally reversed the effect of HU on replication (20,22,35-37). The reversal data indicate that RNR activity per se is not needed for DNA chain elongation. Thus, models in which HU-induced replication arrest is due to inhibition of flux through RNR are not tenable. As long as dNTP levels are maintained above a critical level, RNR activity is dispensable for DNA replication. The results of our dNTP pre-accumulation experiment in yeast (Fig. 2), as well as those of others in mammalian cells (20,22,35-37), are also incompatible with the idea that HU-induced replication arrest is due to complete exhaustion of a privileged dNTP pool that is generated near replication forks and does not equilibrate rapidly with a much larger bulk dNTP pool. If such a privileged dNTP pool existed, it would be quickly depleted when RNR activity was inhibited by HU, and therefore pre-accumulation of dNTPs would not be expected to bypass the HU-induced replication block. Furthermore, rapid equilibration of nuclear and cytosolic dNTPs is suggested by rodent cell experiments that showed that nuclear dNTPs are depleted about as rapidly as cytosolic dNTPs when CHO cells are incubated in HU (3).

The smallest dNTP pool in yeast is dGTP. We determined that α factor-arrested G1 cells contained 312,000 dGTP molecules per cell, or about 10% of the minimal amount theoretically required to replicate the 1.2 x 10^7-bp haploid yeast genome. When cells were released from α factor and allowed to enter S phase in the presence of HU, the dGTP pool fell to a minimum of about 240,000 molecules per cell. Consistent with the pool size reduction being due to DNA replication, the 72,000-molecule decrease in dGTP was roughly matched by a 66,000-molecule decrease in dCTP, and the 114,000-molecule decrease in dATP was roughly matched by a 126,000-molecule decrease in dTTP. If one assumes that the decrease in dNTP levels was due entirely to deoxyribonucleotide incorporation into DNA, HU-treated cells incorporated about 380,000 deoxyribonucleotides before DNA chain elongation became arrested.
Santocanale et al. (31) showed that replication bubbles about 4500 base pairs in length accumulate at two early replication origins when α factor-arrested yeast are released to HU-containing medium. Whole-genome analyses indicated that yeast utilize about 330 efficient origins of replication per S phase (38). If all of these origins fired in the presence of HU, and the resulting elongation complexes incorporated 4500 deoxyribonucleotides per replication fork before arresting, consumption of $3 \times 10^6$ dNTPs per cell would be expected. As we witnessed consumption of only 380,000 dNTP molecules per cell, it suggests that only 13% of yeast origins were of the early type capable of firing in the presence of HU.

Although early replication origins fire in the presence of HU, late replication origins do not (31). Suppression of late origin activity in HU-treated cells requires the checkpoint proteins Rad53 and Mec1 (31). This prompted us to investigate whether dNTP pools became completely exhausted when rad53 cells were released from α factor into medium containing HU. Our results showed that checkpoint-deficient rad53 cells also ceased replication before any dNTP pool became completely exhausted. The degree to which the dNTP pools were affected by HU were roughly the same in wild-type and rad53 yeast. The failure of any dNTP pool to be completely depleted in rad53 cells suggests that the elongation complexes originating from late origins also become arrested shortly after initiation in the presence of HU. Our results indicate that the mechanism sensing dNTP pool levels in HU-treated cells does not require an intact Rad53/Mec1 pathway.

Why would cells evolve a mechanism for arresting replicative DNA elongation before dNTP pools are exhausted? One possibility is DNA damage. It has been shown that excision repair is sensitive to dNTP pool depletion in cultured human fibroblasts (39). Similarly, in yeast expressing an engineered ribonucleotide reductase that is refractive to allosteric dATP feedback inhibition, the resulting increase in dNTP levels is correlated with increased cell survival following DNA damage (19). The conservation of
mechanisms for controlling RNR levels and activity, by the Mec1/Rad53 pathway in yeast and the homologous Atr/Chk2 pathway in mammals (30), suggests that it is important for cells to boost dNTP levels in response to DNA damage. If it takes time for the transcriptional and posttranscriptional processes triggered by the Mec1/Rad53 pathway to boost dNTP levels, it would be advantageous for cells to arrest DNA synthesis at replication forks to conserve the dNTP pools for DNA synthesis at repair sites. According to this view, replication arrest would not only allow time for DNA repair to take place, it would also preserve the activated precursor pools needed for DNA repair. Consistent with this idea, unscheduled DNA synthesis, as measured by [3H]thymidine incorporation in UV-irradiated quiescent fibroblasts, is refractive to HU inhibition (6).

The mechanism for replication arrest when RNR is inhibited remains unknown. If RNR inhibition resulted in significant expansion of the rNTP pools, it is possible that an increased rNTP:dNTP ratio might result in misincorporation of ribonucleotides into DNA and thereby trigger a replication arrest. However, the rNTP pools in yeast, calculated from published data to be 0.34 mM rUTP, 2 mM rATP, 0.26 mM rCTP and 0.34 mM rGTP (40,41), are 5- to 90-fold greater than the corresponding dNTP pools (Fig. 1B and (15, 18)), and are therefore unlikely to be affected by the presence or absence of a relatively small flux of rNDPs through RNR. Indeed, in α factor-synchronized yeast, rNTP levels are constant throughout the cell cycle (19), despite RNR-mediated oscillations in dNTP levels (18,19). Similarly, we observed that the aggregate nucleobase, nucleoside and nucleotide pool, as determined by acid-soluble A260 measurements, did not change during the cell cycle or after treatment with HU (data not shown). Given the large and unchanging size of the rNTP pool, it is unlikely that HU inhibits replication via an effect on rNTP metabolism. Given the observation that dNTP preaccumulation delays the replication arrest (Fig. 2), we feel that the simplest explanation fitting the data is that cells have a mechanism for shutting down replication
before dNTP pools become exhausted. The arrest mechanism does not require an intact 
Rad53/Mec1 pathway, as checkpoint-deficient rad53 cells also do not exhaust their 
dNTP reserves when incubated in HU. It is not clear whether all or a subset of the four 
dNTP pools is sensed. If the smallest pool – dGTP – is the one sensed, DNA elongation 
is arrested when dGTP levels fall below 240,000 molecules per cell. Although we have 
no specific model for the dNTP sensing mechanism, we speculate that in order for 
elongation complexes to progress more than a few thousand base pairs from their point of 
origin, dNTP levels must be maintained above a critical threshold.
REFERENCES


Abbreviations
HU, hydroxyurea; RNR, ribonucleotide reductase.

Acknowledgements
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FIGURE LEGENDS

Fig. 1. DNA synthesis and dNTP levels in α factor-synchronized wild-type yeast released in the absence or presence of HU. DNA content and dNTP levels were determined in wild-type yeast (MY377) that were asynchronously growing (black bar) or were released from an α factor arrest for the indicated number of minutes in the absence (shaded bar) or presence (open bar) of HU. A and C. Flow cytometric analysis of DNA content in the absence or presence of HU, respectively. G1 and G2 peaks are labeled 1C and 2C, respectively. B and D. Analysis of dNTP levels in the absence or presence of HU, respectively. Histograms represent mean ± range for duplicate determinations.

Fig. 2. DNA synthesis and dNTP levels in α factor-synchronized dbf4 cells that were allowed to pre-accumulate dNTPs prior to exposure to HU. Asynchronously growing dbf4 cells (strain MY317) were arrested with α factor and released at the non-permissive temperature (37°C). At 60 min after α factor release, HU was added to indicated cultures (open bar). At 80 min after α factor release, cells were switched to the permissive temperature (25°C). A. Flow cytometric analysis of DNA content. B. Analysis of dNTP levels. Key is the same as in Fig. 1. Histograms represent the average of two time-courses.

Fig. 3. Initiation of replication in the absence of dNTP accumulation. Asynchronously growing dbf4 cells (strain MY317) were arrested with α factor and released to HU at the permissive temperature (25°C). At 50 min after α factor release, cells were switched to the non-permissive temperature (37°C). At 60 min after α factor release, HU was removed. A. Flow cytometric analysis of DNA content. B. Analysis of dNTP levels. Key is the same as in Fig. 1. Histograms represent mean ± range for duplicate determinations.
Fig. 4. DNA synthesis and dNTP levels in α factor-synchronized rad53 yeast released from α factor in the absence or presence of HU. DNA content and dNTP levels were determined in rad53 yeast (strain MY376) that were asynchronously growing (a), α factor-arrested (0), or released from an α factor arrest for the indicated number of minutes. A and C. Flow cytometric analysis of DNA content in the absence or presence of HU, respectively. B and D. Analysis of dNTP levels in the absence or presence of HU, respectively. Key is the same as in Fig. 1. Histograms represent mean ± range for duplicate determinations.
Table 1. Yeast Strains

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Fig. 1

(A) and (C) show DNA content histograms of cells with different treatments. (B) and (D) display the dynamics of nucleotide incorporation under conditions of HU absence and presence, respectively.

- **HU Absent:**
  - dTTP
  - dATP
  - dCTP
  - dGTP

- **HU Added:**
  - dTTP + HU
  - dATP + HU
  - dCTP + HU
  - dGTP + HU

The charts illustrate the amounts of nucleotides incorporated per 10^8 cells over time (min).
Fig. 2

A

B

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pmol nucleotides/10⁸ cells

Time (min)
Fig. 3
Fig. 4

(A) and (C) show the distribution of DNA content with 1C and 2C peaks.

(B) HU absent

- dTTP
- dATP
- dCTP
- dGTP

pmol nucleotides/10^8 cells vs. Time (min)

(D) HU added

- dTTP + HU
- dATP + HU
- dCTP + HU
- dGTP + HU

pmol nucleotides/10^8 cells vs. Time (min)
Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools
Ahmet Koç, Linda J. Wheeler, Christopher K. Mathews and Gary F. Merrill

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