Replication Rapidly Recovers and Continues in the Presence of Hydroxyurea in *Escherichia coli*

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ABSTRACT In both prokaryotes and eukaryotes, hydroxyurea is suggested to inhibit DNA replication by inactivating ribonucleotide reductase and depleting deoxyribonucleoside triphosphate pools. In this study, we show that the inhibition of replication in *Escherichia coli* is transient even at concentrations of 0.1 M hydroxyurea and that replication rapidly recovers and continues in its presence. The recovery of replication does not require the alternative ribonucleotide reductases NrdEF and NrdDG or the translesion DNA polymerases II (Pol II), Pol IV, and Pol V. Ribonucleotides are incorporated at higher frequencies during replication in the presence of hydroxyurea. However, they do not contribute significantly to the observed synthesis or toxicity. Hydroxyurea toxicity was observed only under conditions where the stability of hydroxyurea was compromised and by-products known to damage DNA directly were allowed to accumulate. The results demonstrate that hydroxyurea is not a direct or specific inhibitor of DNA synthesis *in vivo* and that the transient inhibition observed is most likely due to a general depletion of iron cofactors from enzymes when 0.1 M hydroxyurea is initially applied. Finally, the results support previous studies suggesting that hydroxyurea toxicity is mediated primarily through direct DNA damage induced by the breakdown products of hydroxyurea, rather than by inhibition of replication or depletion of deoxyribonucleotide levels in the cell.

IMPORTANCE Hydroxyurea is commonly suggested to function by inhibiting DNA replication through the inactivation of ribonucleotide reductase and depleting deoxyribonucleoside triphosphate pools. Here, we show that hydroxyurea only transiently inhibits replication in *Escherichia coli* before replication rapidly recovers and continues in the presence of the drug. The recovery of replication does not depend on alternative ribonucleotide reductases, translesion synthesis, or RecA. Further, we show that hydroxyurea toxicity is observed only in the presence of toxic intermediates that accumulate when hydroxyurea breaks down, damage DNA, and induce lethality. The results demonstrate that hydroxyurea toxicity is mediated indirectly by the formation of DNA damage, rather than by inhibition of replication or depletion of deoxyribonucleotide levels in the cell.

KEYWORDS DNA replication, RNase H, hydroxyurea, translesion DNA synthesis
targets and inactivates ribonucleotide reductase. In vitro, several studies have demonstrated that addition of hydroxyurea to the purified enzyme or to cell extracts inactivates ribonucleotide reductase activity in both prokaryotes and eukaryotes (6, 16, 17). However, whether this is the mechanism that operates in vivo is less clear. Some early studies observed that hydroxyurea reduced dNTP concentrations immediately after addition in Escherichia coli cultures (10, 18). However, other studies found that hydroxyurea’s effects on the cell were not specific to dNTP concentrations and that transcription, translation, and growth were also inhibited to various degrees (19, 20). Similarly, some studies in eukaryotes have found that hydroxyurea depletes cellular concentrations of dNTPs (21, 22), whereas others found dNTP levels remained largely unaffected by the presence of hydroxyurea (23, 24). In E. coli, the primary ribonucleotide reductase, essential for aerobic growth, is a two-subunit, iron-dependent, class Ia enzyme encoded by nrdA and nrdB. The larger R1 subunit (NrdA) contains allosteric ATP- and nucleotide-binding sites, as well as the active site required for catalysis of ribonucleotide reduction, whereas the smaller R2 subunit (NrdB) contains a di-iron center that is required for initiation of NrdA activity (25). E. coli also encodes two alternative ribonucleotide reductases—NrdEF, a manganese-dependent class Ib ribonucleotide reductase that is active when iron is limiting (26), and NrdDG, a class III enzyme that is required for growth under anaerobic conditions (27).

Ribonucleotide reductase is required for the de novo synthesis of dNTPs through a two-step reaction sequence that depends on free radical chemistry to reduce the 2’ carbon of a ribonucleotide to its deoxyribonucleotide derivative (28). In E. coli, generation of a stable tyrosyl free radical in the R2 subunit occurs following conversion of the NrdB protein from its ferrous to its ferric form by molecular oxygen. Radical transfer from NrdB to a cysteine residue on NrdA then initiates catalysis of the ribonucleotide reduction reaction when substrate ribonucleotide and the allosteric effector ATP are bound to the R1 subunit (25, 28). In vitro, hydroxyurea inactivates ribonucleotide reductase by scavenging the iron-stabilized tyrosyl free radical from the active site of the R2 subunit (6, 8).

Alternatively, inactivation of ribonucleotide reductase by hydroxyurea may simply reflect a broad effect that the drug has on enzymes containing catalytic metals. Recent studies have found that hydroxyurea alters Fe-S centers that act as cofactors for various cellular enzymes (11–13). Hydroxyurea can also form complexes with iron and copper directly and may act as a weak chelator in vivo (29, 30). Many Fe-S center proteins catalyze redox reactions that are required to maintain normal metabolism in the cell, suggesting that hydroxyurea could inhibit replication through a generalized effect on proteins requiring catalytic metals, of which NrdAB is one. This type of explanation would be consistent with many studies where inhibition of several processes was observed, including replication, transcription, and translation, and required extremely high hydroxyurea concentrations of 0.1 to 0.2 M (9, 19, 20, 31).

A third proposed mechanism of action for hydroxyurea suggests its toxicity is mediated by direct induction of DNA damage. The stability of hydroxyurea in its aqueous form is compromised over time or in the presence of heat and breaks down to form N-hydroxyurethane and carbamoyloxyurea, as well as hydrogen cyanide, nitric oxide, and peroxide (15, 32). Incubating hydroxyurea at temperatures of 37°C and above was found to generate nitrosourea, o-carbamoylhydroxylamine, and carbamoyloxyurea (32), while stocks of hydroxyurea stored at 37°C for several days were observed to contain nitric oxide and hydrogen cyanide (15). These agents can react with DNA, inducing adducts and base damage. Hydroxyurea has also been proposed to induce oxidative free radicals that damage DNA through interactions with iron or other metals (14, 15, 33). Hydroxyurea treatment in E. coli cells has been indirectly shown to induce the formation of superoxide, which can subsequently be converted to hydrogen peroxide and react with iron to produce DNA-damaging hydroxyl radicals (33). Additionally, in vitro studies have correlated the formation of 8-hydroxy-2’-deoxyguanosine lesions with the production of nitric oxide in aqueous solutions of hydroxyurea that also contain copper (14).
Mutations in several replication and repair genes have been shown to affect survival in the presence of hydroxyurea. Loss of translesion DNA polymerases IV (Pol IV) and Pol V has been reported to confer modest resistance to hydroxyurea (34). Conversely, recA mutants and xthA nfo AP endonuclease mutants are reported to be hypersensitive to hydroxyurea (15, 35, 36). These phenotypes have been proposed to be produced by a mechanism of action that could be consistent with either inhibition of replication or induction of DNA damage.

Thus, to further characterize hydroxyurea’s mechanism of action, we characterized its inhibitory effect on the rate of DNA synthesis directly in E. coli. We show that hydroxyurea only transiently inhibits DNA synthesis in vivo and that this brief inhibition occurs only when high (0.1 M) concentrations of drug are used. Following inhibition, DNA synthesis recovers and is able to continue for several hours in the presence of the drug. Using recA mutants as an indicator for the ability to replicate in the presence of DNA damage, we show that hydroxyurea toxicity is observed only under conditions where the stability of hydroxyurea is compromised and toxic intermediates known to damage DNA directly are allowed to accumulate.

RESULTS

DNA replication in E. coli is only transiently inhibited by acute exposure to hydroxyurea. Hydroxyurea is commonly thought to inhibit ongoing DNA replication indirectly by targeting the class IIa ribonucleotide reductase (nrdAB gene products) of E. coli and depleting the dNTP pools required for DNA synthesis to occur (5, 6, 10). However, how hydroxyurea affects the rate of DNA synthesis in vivo has never been characterized directly. To examine this, we monitored the rate of DNA synthesis following exposure to 1, 10, and 100 mM hydroxyurea by incubating [14C]thymine-prelabeled cultures with [3H]thymidine for 2 min at various times after drug addition. The rate of DNA synthesis ([3H] incorporation/2 min) could then be determined relative to the total amount of DNA present ([14C] incorporation) at specific times following treatment. In each case, we included a mock-treated control to directly compare exposed cultures to unexposed cultures and to ensure that any changes in the observed rates were due to the treatment rather than to culture density.

Based on the predicted mode of action, we expected that following the addition of hydroxyurea, DNA synthesis would be inhibited for the duration of the time course. Surprisingly, this is not what we observed. Following drug addition, an initial and rapid decrease in the rate of DNA synthesis that varied directly as a function of the hydroxyurea dose was observed (Fig. 1). The rate of synthesis initially decreased by <10%, ~70%, and >90% in the presence of 1, 10, and 100 mM hydroxyurea, respectively. However, in each case, DNA synthesis began to rapidly recover after 5 min in the presence of the drug, and replication was fully restored within the 60-min time course even in the presence of 100 mM hydroxyurea. The total DNA accumulation in each culture was reduced in a manner that correlated with the rate of DNA synthesis observed in each case. The rate of DNA synthesis and total DNA accumulation were reduced by averages of 3% and 0%, 23% and 40%, and 68% and 80% for 1, 10, and 100 mM hydroxyurea, respectively.

The recovery of DNA synthesis in the presence of hydroxyurea was unexpected given the proposed inhibitory activity of the drug on ribonucleotide reductase. The results indicate that the inhibition of replication by hydroxyurea requires high concentrations of the drug and that cells have mechanisms that allow replication to rapidly resume in its presence.

The recovery of replication in the presence of hydroxyurea is not mediated by alternative ribonucleotide reductases or translesion DNA polymerases. NrdAB is the primary ribonucleotide reductase that operates under aerobic conditions. However, E. coli carries genes, nrdEF and nrdDG, that encode two alternative ribonucleotide reductases that each function under different growth conditions (26, 27) and that are transcriptionally upregulated following hydroxyurea treatment (33). Thus, it is possible that these alternative ribonucleotide reductases, NrdEF and NrdDG, are insensitive to
hydroxyurea and compensate for NrdAB, thereby allowing replication to resume. To examine this directly, we constructed isogenic mutants lacking the cryptic class Ib ribonucleotide reductase NrdEF and the class III ribonucleotide reductase-activating enzyme NrdG. The abilities of these mutants to recover replication in the presence of 100 mM hydroxyurea were then compared to that of wild-type cells. As shown in Fig. 2A, the rate of DNA synthesis decreased to the same extent and recovered with similar kinetics in wild-type, nrdEF, and nrdG cultures. These results indicate that the recovery of DNA synthesis in the presence of hydroxyurea does not depend on the alternative ribonucleotide reductase NrdEF or NrdDG. However, we were unsuccessful in our attempts to construct an nrdEF nrdDG double mutant, perhaps suggesting a requirement for at least one of these ribonucleotide reductases at some stage of growth on plates, in culture, or during strain construction.

We next considered the possibility that recovery was mediated through the translesion DNA polymerases. E. coli’s three translesion DNA polymerase genes are transcriptionally upregulated in cultures challenged with hydroxyurea (33). Furthermore, Pol IV and Pol V mutants are reported to be more resistant to hydroxyurea than wild-type cells, and the translesion DNA polymerases have been speculated to catalyze error-prone DNA synthesis when dNTP pools are low or unbalanced (34). However, when we examined the abilities of mutants lacking all three translesion DNA polymerase genes (polB dinB umuDC mutants) to recover replication in the presence of hydroxyurea, we observed no difference in the recovery rates of these mutants compared to those of wild-type cells (Fig. 2B). Similar to wild-type cells, the rate of DNA synthesis in hydroxyurea-treated polB dinB umuDC mutants decreased by more than 90% in the first 10 min following hydroxyurea addition, began to recover at 20 min, and approached untreated levels by 50 min. The results demonstrate that the translesion DNA polymerases are not responsible for the replication occurring in the presence of hydroxyurea.

Ribonucleoside incorporation increases but does not account for the synthesis observed in the presence of hydroxyurea. Ribonucleoside triphosphate (rNTP) con-
centrations found in both eukaryotic and prokaryotic cells exceed those of dNTPs by 10- to 100-fold under normal growth conditions (37–39), leading to misincorporation of rNTPs into genomic DNA by polymerases at rates that have been estimated to be ~1 rNTP per 2.3 kb (38, 39). Treatment with hydroxyurea further increases ribonucleoside diphosphate (rNDP) levels and decreases dNTP pools by as much as 10-fold over untreated cells (10), raising the possibility that the synthesis occurring in the presence of hydroxyurea could contain significant amounts of ribonucleotides. In E. coli, single ribonucleoside monophosphate (rNMP) residues in genomic DNA are removed by the enzyme RNase HII (encoded by \( rnhB \)) while long RNA-DNA tracts, like those found in Okazaki fragments, are cleaved by RNase HI (encoded by \( rnhA \)) (40, 41).

To examine whether rNTP misincorporation might contribute to the observed synthesis in the presence of hydroxyurea, we initially constructed isogenic mutants lacking \( rnhB \) and \( rnhA \) and monitored overall DNA replication and rates of synthesis as described previously. If the frequency of rNTP incorporation in DNA increased with hydroxyurea treatment, we predicted that in the absence of RNase HI or RNase HII these ribonucleotides would not be degraded or removed, potentially resulting in more \(^3\)H and \(^14\)C incorporation in these mutants than in wild-type cells. Contrary to this prediction, we observed that the time and kinetics of replication resumption in \( rnhB \) cultures were similar to those in wild-type cells following hydroxyurea addition (Fig. 3A). In \( rnhA \) mutants, the absence of RNase HI affected the rate, but not the time, at which DNA synthesis recovered after hydroxyurea addition. The overall rate of DNA synthesis was much reduced in hydroxyurea-treated \( rnhA \) cells compared to the wild-type parent; however, this difference could be attributed to \( rnhA \)'s slow-growth phenotype and its role in Okazaki primer removal (41) and is reflected in the reduced DNA accumulation seen in mock-treated \( rnhA \) mutants (Fig. 3A).

To determine if extensive ribonucleotide incorporation occurs during growth in hydroxyurea, cultures of wild-type and \( rnhA \) and \( rnhB \) mutant cells were allowed to

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**FIG 2** Cryptic class Ib and class III ribonucleotide reductases and translesion DNA polymerases do not contribute to the recovery of DNA replication in the presence of hydroxyurea. \(^3\)H thymidine was added to \(^14\)C thymine-prelabeled cultures for 2 min at the indicated times following treatment with 100 mM hydroxyurea (filled symbols) or mock treatment (open symbols) at time zero. The total DNA accumulation (\(^14\)C; circles) and the rate of DNA synthesis (\(^3\)H; squares) are plotted for wild-type (WT), \( nrdEF \) (class I ribonucleotide reductase), and \( nrdG \) (class III ribonucleotide reductase-activating enzyme) (A) and \( polB \) \( dinB \) \( umuDC \) (B) cells. Each graph represents an average from at least two independent experiments. The error bars represent one standard error of the mean.
replicate in the presence or absence of 100 mM hydroxyurea for 4 h, a period of time in which DNA more than doubles (Fig. 3B). Following this period, the genomic DNA was purified and incubated with 200 mM NaOH to hydrolyze the DNA backbone at rNMP moieties. The samples were then analyzed by denaturing alkali agarose gel electrophoresis. In wild-type cultures, genomic DNA purified and treated in this manner migrated with a size greater than 40 kb (Fig. 3B). Following 2 or 4 h of replication in hydroxyurea, the DNA remained essentially intact, and very little DNA fragmentation was observed following alkali denaturation, indicating that few ribonucleotides were present in the DNA. DNA similarly remained intact in rnhA mutants throughout the time.
course, suggesting that long stretches of ribonucleotides were not incorporated or removed by RNase HI during hydroxyurea incubation. In contrast, significant ribonucleotide incorporation was observed in rnhB mutants. Although the genomic DNA of rnhB mutants was of high molecular weight when purified from cells before the addition of hydroxyurea, a significant loss of this high-molecular-weight DNA was observed at both 2 and 4 h after incubation in hydroxyurea (Fig. 3B). The loss of high-molecular-weight DNA was accompanied by the appearance of smaller fragments ranging in size between 4 and 40 kb. This observation demonstrates that during replication in hydroxyurea, elevated levels of ribonucleotides are incorporated and removed by RNase HII. The frequency of ribonucleotide incorporation in the presence of hydroxyurea remained below 1 ribonucleotide per several thousand bases, arguing that it is unlikely to account for the observed synthesis that occurs under these conditions.

Based on the above interpretation, we again monitored the overall DNA replication and rates of synthesis in wild-type cultures but this time treated each of the lysed cell aliquots with 500 mM NaOH to remove any ribonucleotides prior to precipitation with trichloroacetic acid. Trichloroacetic acid effectively precipitates polynucleotides longer than 12 bp. We reasoned that if significant levels of ribonucleotides were incorporated during recovery, the amount of precipitable nucleotides should be detectably reduced in the alkali-treated samples. However, as shown in Fig. 3C, no difference was observed in the rate of recovery or amount of DNA synthesis when alkali-treated and non-alkali-treated samples were compared. Thus, although the frequency of ribonucleotide incorporation increases during incubation in hydroxyurea, it cannot account for the synthesis occurring under these conditions.

Transient inhibition by hydroxyurea pauses, but does not disrupt, replication, and recovery occurs independently of RecA. To further characterize the nature of the transient replication inhibition seen after hydroxyurea addition, we examined whether the recovery of DNA synthesis depended upon RecA. Cells exposed to hydroxyurea upregulate recA gene expression as part of the SOS response (33, 42), and RecA is required for replication to recover following disruption by DNA damage (reviewed in reference 43). To determine if the recovery of replication after hydroxyurea inhibition also required RecA, we examined the replication of recA cells in the presence of 100 mM hydroxyurea. As a control, and for the purpose of comparison, we also monitored the rate of replication in recA mutants following either UV irradiation or treatment with hydrogen peroxide. In both UV-irradiated and hydrogen peroxide-treated wild-type cultures, DNA synthesis was transiently inhibited before it was seen to recover (Fig. 4A and B). The time at which DNA synthesis recovers following UV or hydrogen peroxide treatment has been shown to correlate with the removal of the blocking lesions from the template through a process that is coupled with replication (44–49). In comparison, in recA mutants, no further DNA synthesis was observed after either UV irradiation or treatment with hydrogen peroxide, consistent with RecA’s role in restoring replication after disruption by DNA damage (Fig. 4A and B).

In contrast to UV irradiation or hydrogen peroxide treatment, both wild-type and recA cells resumed DNA synthesis with similar kinetics following the addition of hydroxyurea (Fig. 4C). DNA replication continued in the absence of RecA even when the time course was extended to 6 h (Fig. 4D). In both wild-type and recA mutant cultures, hydroxyurea treatment resulted in a modest, ~50% reduction in DNA accumulation over this period. We interpret these results to imply that the transient inhibition caused by hydroxyurea stalls or pauses the replisome but does not disrupt its integrity, as replication recovery occurs independently of RecA. This observation also implies that no direct DNA damage is formed under these conditions and is most consistent with the inhibitory effect of hydroxyurea resulting from a more global disruption of iron-dependent enzymes. Consistent with this interpretation, an inhibitory effect on transcription was also observed in the presence of hydroxyurea (see Fig. S1 in the supplemental material). In contrast to the case of DNA synthesis, no recovery of transcription was observed during the time course, suggesting that disruption of iron-dependent transcriptional enzymes is perhaps irreversible and requires new protein synthesis. Similarly, other studies have
documented that several metabolic processes are inhibited in the presence of hydroxyurea (9, 19, 20, 31). Taken together, the observations further support the idea that HU affects a broad range of metabolic processes in the cell.

**RecA contributes to survival and is required for the recovery of replication only under conditions when the stability of hydroxyurea is compromised and toxic intermediates accumulate.** The lack of a requirement for RecA in order to recover replication in the presence of hydroxyurea was unexpected, as we and others had observed that recA mutants exhibit reduced viability when spotted on agar plates containing hydroxyurea (Fig. 5A) (36). In surveying the literature, we noticed that the concentration of hydroxyurea required to reduce viability varied drastically between studies and appeared to depend primarily on whether the assay was done on solid medium, such as agar plates, or in liquid culture. Whereas 5 to 10 mM was sufficient to reduce viability using hydroxyurea on agar plates, 100 to 200 mM concentrations were required when liquid cultures were used (15, 31, 33, 34, 36, 50). This discrepancy may arise due to the instability of hydroxyurea. Previous studies have found that hydroxyurea breaks down into toxic intermediates that include N-hydroxyurethane and carbamoyloxyurea, as well as hydrogen cyanide, nitric oxide, or peroxides, over time or when exposed to heat (14, 15, 32). Since both time and heat exposure are required to prepare hydroxyurea-containing agar plates, we reasoned that the toxicity observed previously on solid media might be explained by the breakdown of hydroxyurea into these toxic by-products that are known to damage DNA. To test this idea directly, we incubated hydroxyurea at 37°C for 48 h and then examined the survival of wild-type and recA cultures treated with the agent in liquid cultures. To this end, wild-type and recA cultures grown to early exponential phase (optical density at 600 nm [OD$_{600}$] = 0.3) were divided and treated with 100 mM fresh or 2-day heat-decayed preparations of hydroxyurea. The viability of each culture was then followed over time. Whereas, wild-type cultures maintained viability under both conditions, recA cultures lost viability.
when treated with the 2-day-old preparations of hydroxyurea. The recA cultures were not sensitive to the freshly prepared hydroxyurea (Fig. 5B).

We then examined the effect that the 2-day heat-decayed preparation of hydroxyurea had on replication. In contrast to the fresh preparation, the 2-day-old preparation of hydroxyurea significantly impaired the abilities of both the wild-type and the recA cultures to restore DNA synthesis following exposure (Fig. 5C). The observations argue that the primary cause of hydroxyurea toxicity is direct induction of DNA damage by toxic by-products of hydroxyurea that accumulate over time. Consistent with this interpretation, processing of hydroxyurea by the endogenous catalases (encoded by katE and katG) of E. coli produces the nitric oxide intermediates responsible for hydroxyurea toxicity in plates, similar to what is observed in vitro and in Arabidopsis (51, 52). Inactivation of both classes of catalases prevents this processing.
and efficiently suppresses the hypersensitivity of recA mutants to hydroxyurea in plates (see Fig. S2A in the supplemental material). Importantly however, the time of replication recovery in the presence of fresh hydroxyurea is unaffected by the absence of catalases, even in a recA mutant (see Fig. S2B in the supplemental material). The results strongly argue that free radical DNA damage is not associated with the observed transient inhibition of replication after hydroxyurea treatment. To determine whether the restoration of DNA synthesis requires transcription or protein synthesis, we examined whether the recovery would occur in the presence of either rifampin or chloramphenicol, which inhibit transcription and translation, respectively. As shown in Fig. S3 in the supplemental material, DNA synthesis appeared to begin to recover in the absence of either transcription or new protein synthesis. However, since new rounds of DNA replication from oriC also require transcription and translation, the rate of DNA synthesis declined rapidly in both mock-treated samples in the presence of either rifampin or chloramphenicol. Thus, we cannot rule out the possibility that new protein synthesis is not required to observe a complete recovery of DNA synthesis in the presence of hydroxyurea.

Finally, we examined the survival of nrdEF, nrdG, polB dinB umuDC, mnhA, and mnhB mutants when exposed to hydroxyurea on solid media to determine if the products of these genes contributed to survival and were needed for either the recovery of replication or the repair of hydroxyurea-induced lesions. To this end, we propagated wild-type and nrdEF, nrdG, polB dinB umuDC, mnhA, mnhB, and recA mutant cells in minimal medium to early exponential phase (OD$_{600}$ = 0.3) and then serially diluted the cultures and plated them on solid medium containing increasing concentrations of hydroxyurea. As shown in Fig. 5D, whereas the recA mutant exhibited extreme hypersensitivity to hydroxyurea under these conditions, none of the other mutants were hypersensitive. Taken together, the results indicate that the cryptic class Ib ribonucleotide reductase (NrdEF), the class III ribonucleotide reductase (NrdDG), the translesion DNA polymerases, RNase HI (mnhA), and RNase HII (mnhB) are not required for the recovery of replication or the repair of hydroxyurea-induced DNA damage.

**DISCUSSION**

Hydroxyurea treatment has been proposed to inhibit DNA replication through the targeted inhibition of ribonucleotide reductase, eventually leading to cell death. The results presented here show that when cultures are treated with high concentrations of hydroxyurea, DNA synthesis is only transiently inhibited, and no loss of viability is associated with this inhibition. The recovery of replication occurs even in the absence of RecA, arguing that the initial inhibition caused by hydroxyurea pauses, rather than disrupts, the replication machinery at the fork. Following the brief period of inhibition, replication resumes and continues, even in the presence of hydroxyurea.

We tested the idea that reduction of rNTPs to dNTPs by *E. coli*’s two alternative ribonucleotide reductases might account for the resumption of DNA synthesis in the presence of hydroxyurea. Both the class I and class III ribonucleotide reductases are transcriptionally upregulated in response to the drug (33), making this a reasonable hypothesis. However, we found no evidence for this in our study. nrdEF and nrdG mutants restored DNA replication rates at the same time and with kinetics similar to those of wild-type cells after hydroxyurea treatment. The restoration of DNA replication also did not depend on the translesion DNA polymerases. In the presence of hydroxyurea, polB dinB umuDC mutants recovered DNA synthesis rates as well as the wild-type parent. *Saccharomyces cerevisiae* DNA polymerase ζ and *E. coli* DNA polymerase V have been shown to alter mutagenic frequencies following hydroxyurea treatment (34, 53). However, in light of the results presented here, this effect seems likely to be due to DNA damage formed by by-products of hydroxyurea, rather than an effect associated with restoring synthesis at paused forks.

The data we present demonstrate that extensive incorporation of rNTPs is not responsible for the recovery of DNA synthesis observed in the presence of hydroxyurea. Although elevated levels of ribonucleotides were detected in mutants lacking RNase
hydroxyurea was indistinguishable from that observed following UV irradiation, con-

terior of inhibition and recovery (13) of reactive oxygen species or Fenton chemistry inducing DNA damage during this initial examination. Although hydroxyurea affects iron regulation, we did not see evidence of homeostasis and could explain the lack of phenotype for any of the mutants we examined. While hydroxyurea is specific to ribonucleotide reductase. Rather, these effects would be more consistent with the general depletion of Fe-S centers in enzymes required for metabolism. Hydroxyurea has been shown to alter the Fe-S centers of enzyme cofactors at concentrations required to induce the inhibition, it seems unlikely that the target of study. Considering the rapid and transient nature of this inhibition and the high normal levels within the 60-min recovery time of the experiments performed in this study. Considering the rapid and transient nature of this inhibition and the high concentrations required to induce the inhibition, it seems unlikely that the target of hydroxyurea is specific to ribonucleotide reductase. Rather, these effects would be more consistent with the general depletion of Fe-S centers in enzymes required for metabolism. Hydroxyurea has been shown to alter the Fe-S centers of enzyme cofactors involved in yeast redox reactions (13); to target several metalloproteins in higher eukaryotes (reviewed in reference 11); and to inhibit PriL, an Fe-S containing replication enzyme, in Sulfolobus (12), suggesting a more generalized effect of the drug on proteins requiring catalytic metals, of which NrdAB is one. In addition, hydroxyurea is capable of forming complexes with iron and copper in vitro, potentially acting as a weak chelator (29, 30). Iron is required in a variety of biological processes, and it is possible that partial chelation of these metal ions by hydroxyurea triggers a switch in cellular metabolism. Intracellular iron levels in E. coli are transcriptionally regulated by Fur repression, which uses Fe^{2+} as a corepressor (58). When iron levels are low, Fur modulates the expression of iron metabolism genes involved in iron transport and storage, decreases expression of iron-containing respiratory proteins, such as cytochrome oxidases (products of cyoABCDE and cydAB), and also appears to affect the expression of genes involved in diverse cellular processes, such as glycolysis, purine metabolism, and redox stress (58). Interestingly, E. coli treated with hydroxyurea upregulated the expression of iron transport genes and downregulated cytochrome b (cybB) (33), much like what is seen under low-iron growth conditions, supporting the idea that hydroxyurea induces an iron-specific cellular response. In this context, the results of our study suggest that the transient decline and quick recovery of replication rates following hydroxyurea treatment are products of a global sensing mechanism that E. coli uses for metal ion homeostasis and could explain the lack of phenotype for any of the mutants we examined. Although hydroxyurea affects iron regulation, we did not see evidence of reactive oxygen species or Fenton chemistry inducing DNA damage during this initial period of inhibition and recovery (13, 15, 33, 50), as recA mutants, which fail to replicate in the presence of DNA damage, replicated and survived as well as wild-type cells when treated with hydroxyurea. In contrast, if hydroxyurea was stored under conditions that affected its stability (15, 32), then the recovery of replication and viability became dependent on recA. The behavior of replication in recA mutants treated with the breakdown products of hydroxyurea was indistinguishable from that observed following UV irradiation, con-
sistent with the presence of DNA damage. These observations are consistent with work by Kuong and Kuzminov, who showed that the effect of “aged” hydroxyurea on cell survival was more severe than that of freshly prepared hydroxyurea and was correlated with the accumulation of hydrogen cyanide, nitric oxide, and hydrogen peroxide (15).

The DNA damage induced when using heat-exposed or old stocks of hydroxyurea may also resolve why hydroxyurea is so much more potent as a toxin when cells are treated with hydroxyurea that has been incorporated into agar plates than with direct addition of the drug to liquid cultures. Hydroxyurea incorporated into plates is at least 2 days old and has been exposed to temperatures above 50°C, allowing these breakdown products known to damage DNA to accumulate. These differing conditions also likely explain discrepancies in previous studies with respect to the hypersensitive phenotype of various repair mutants (15, 36, 59, 60).

We believe these results are most consistent with the idea that hydroxyurea added to growing *E. coli* cultures transiently disrupts metabolism by inactivating enzymes requiring transition metal chemistry (Fig. 6). These include the primary ribonucleotide reductase NrdAB, as well as other targets that are required to maintain ongoing DNA synthesis, transcription, translation, and cell growth (12, 13, 58, 61). Once the iron balance is restored or compensated for, replication can continue for several hours. Toxicity from hydroxyurea is only observed after several hours and is likely to be due to DNA damage induced by toxic intermediates that have been reported to accumulate as hydroxyurea breaks down.

**MATERIALS AND METHODS**

**Bacterial strains.** The bacterial strains used in this study are summarized in Table 1. All the strains are derived from SR108, a thyA36 deoC2 derivative of W3110 (62). HL921 [SR108 Δ(srl-recA306::Tn10), CL646 (SR108 polB::F1 Sm-Sp dinB::Kan’ [kanamycin resistance] umuDC395::cat), and CL854 (SR108 uvrA::Tn10 recA::cat)] have been previously described (44, 63, 64). CL2602 (SR108 nrdG::Kan’) was constructed by P1 transduction of the nrdG784::Kan’ allele from JW4196-3 into SR108 (65). CL3360 (SR108 mphB2::Kan’ [Kanamycin resistance]) was constructed by P1 transduction of the mphB::Kan’ allele from JW0178 into SR108 (65). CL3362 (SR108 mphA::Kan’) was constructed by P1 transduction of the mphA733::Kan’ allele from JW0204 into SR108 (65).

CL2164 (DY329 mphEF::cat) was constructed by gene replacement using the recombineering strain DY329 (66). The cat cassette was amplified from CL646 using the nrdE:camF primer (5’-CTCATGGGTAC GCAAACGCTATCGAAACGGTAGTGACTGGAAAGATGACCTCGG-3’) and the nrdF:camR primer (5’-GCGTGATAAAAAGCTATTTGGCGGGAATTATTTCCCTGCTGACCAGCAATGACATAAAGCG-3’). The PCR product was transformed into DY329 to generate CL2164, selecting for chloramphenicol resistance. The gene replacement was then moved into SR108 by standard P1 transduction, generating strain CL2581 (SR108 mphEF::cat).

CL1406 (DY329 katE::FRT-cat-FRT) was constructed by gene replacement using the recombineering strain DY329. The cat cassette was amplified from pKD3 (67) using the katEF-FRT CamF primer (5’-TTGATGAAATTAAAGGAGACGGTAGTCCACTAGTAGTGAGCTGAGCTGCTC-3’) and the katER-FRT CamR primer (5’-GCGCAGATTTGGCAGGCTGCGATTTCCCCTTCCCGGCAAATGACATAAAGCG-3’). The PCR product was transformed into DY329 to generate CL1406, selecting for chloramphenicol resistance.
resistance. The gene replacement was then moved into SR108 by standard P1 transduction, generating strain CL1420 (SR108 katE::FRT [FLP recombinase target]-cat-FRT). The chloramphenicol resistance cassette was then eliminated from CL1420 using the temperature-sensitive plasmid pCP20 (68) encoding FLP recombinase to generate CL1424 (SR108 katE::FRT).

CL1408 (DY329 katG::FRT-cat-FRT) was constructed by gene replacement using the recombinering strain DY329. The cat cassette was amplified from pKD3 using the katG-FRTCam primers (5′-AGCGTA ACCATGATGAGGGGACACATTTAGGAGGCACGTGCCTGAGGTGCATAGATATCCTGTTA). The PCR product was transformed into DY329 to generate CL1408, selecting for chloramphenicol resistance. The gene replacement was then moved into CL1424 by standard P1 transduction, generating strain CL1429 (SR108 katE::FRT katG::FRT-cat-FRT). The chloramphenicol resistance cassette was then eliminated from CL1429 using the temperature-sensitive plasmid pCP20 encoding FLP recombinase to generate CL1438 (SR108 katE::FRT katG::FRT). CL1440 (SR108 katE::FRT katG::FRT recA::cat) was constructed by P1 transduction of the recA::cat allele from CL854 into CL1438.

**DNA synthesis and accumulation.** UV irradiation used a 15-W germicidal lamp (254 nm) at an incident dose of 0.9 J/m²/s. For experiments using UV irradiation, fresh overnight cultures were diluted 1:100 and grown at 37°C in Davis medium [2.0 g KH₂PO₄, 7.0 g KH₂PO₄, 0.5 g Na₂C₁₂H₁₇O₂₄, 0.1 g MgSO₄·6H₂O, 1.0 g (NH₄)₂SO₄ per liter, pH 7.0] supplemented with 0.4% glucose, 0.2% Casamino Acids, 10 μg/ml thymine (DGChy medium) and 0.1 μCi/ml [¹⁴C]thymine to an OD₆₀₀ of precisely 0.3. At that time, half of the cells were mock irradiated, while the remaining culture was UV irradiated with an incident dose of 27 J/m².

Fresh stocks of 1 M hydrogen peroxide were prepared by diluting 50% hydrogen peroxide (14.7 M) in deionized water immediately before experimental use. For experiments using hydrogen peroxide, overnight cultures were diluted 1:100 and grown at 37°C in LB medium supplemented with 10 μg/ml thymine (LBthy) and 0.1 μCi/ml [¹⁴C]thymine to an OD₆₀₀ of precisely 0.3, at which point half of the cells were mock treated while the remaining culture was treated with 10 mM hydrogen peroxide for 5 min at 37°C. Following either mock or hydrogen peroxide treatment, catalase (Fisher brand) was added directly to the culture to a final concentration of 200 μg/ml to remove excess hydrogen peroxide from the medium.

Fresh stocks of 2 M hydroxyurea were prepared in deionized water immediately before experimental use. To prepare heat-decayed hydroxyurea, a 2 M stock of hydroxyurea in deionized water was made and stored in an air-tight tube sealed with Parafilm for 48 h at 37°C. For experiments using hydroxyurea, overnight cultures were diluted 1:100 and grown at 37°C in DGChy supplemented with 0.1 μCi/ml [¹⁴C]thymine to an OD₆₀₀ of precisely 0.3, at which point one-fourth of the cells were mock treated while the remaining culture was divided equally and exposed to 1 mM, 10 mM, and 100 mM freshly prepared hydroxyurea to determine the optimum dose. In subsequent experiments, cells were subcultured and grown at 37°C in DGChy supplemented with 0.1 μCi/ml [¹⁴C]thymine to an OD₆₀₀ of precisely 0.3, at which point half of the cells were mock treated while the remaining culture was treated with 100 mM hydroxyurea that was freshly prepared or heat treated.

### Table 1. E. coli K-12 strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR108</td>
<td>λ-thyA36 deoC2 In(rrmD-rrmE1) rph</td>
<td>62</td>
</tr>
<tr>
<td>DY329</td>
<td>ΔlacU169 adaA::Tn10 galD40 lacI857 Δ(cro-bioA)</td>
<td>66</td>
</tr>
<tr>
<td>JW1946-3</td>
<td>nrdG784::Kan’</td>
<td>65</td>
</tr>
<tr>
<td>JW0178</td>
<td>mII872::Kan’</td>
<td>65</td>
</tr>
<tr>
<td>JW2040</td>
<td>mIIA733::Kan’</td>
<td>65</td>
</tr>
<tr>
<td>CL2164</td>
<td>ΔlacU169 adaA::Tn10 galD40 lacI857 Δ(cro-bioA) nrdE::cat</td>
<td>DY329 × PCR fragment (nrdE-cat and nrdF-cat primes)</td>
</tr>
<tr>
<td>CL1406</td>
<td>ΔlacU169 adaA::Tn10 galD40 lacI857 Δ(cro-bioA) katE::FRT</td>
<td>CL1408 × PCR fragment (katE-FRTCamF and katER-FRTCamR primes)</td>
</tr>
<tr>
<td>CL1408</td>
<td>ΔlacU169 adaA::Tn10 galD40 lacI857 Δ(cro-bioA) katG::FRT-cat-FRT</td>
<td>CL1408 × PCR fragment (katG-FRTCamF and katGR-FRTCamR primes)</td>
</tr>
<tr>
<td>Strains isogenic to SR108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL921</td>
<td>recA::Tn10</td>
<td>44</td>
</tr>
<tr>
<td>CL646</td>
<td>polB::11 Sm-Sp dinA::Kan’ umuDC595::cat</td>
<td>63</td>
</tr>
<tr>
<td>CL854</td>
<td>uvrA::Tn10 recA::cat</td>
<td>64</td>
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<tr>
<td>CL2581</td>
<td>nrdE::cat</td>
<td>SR108 × P1 (CL1264)</td>
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<tr>
<td>CL2602</td>
<td>nrdG784::Kan’</td>
<td>SR108 × P1 (JW1946-3)</td>
</tr>
<tr>
<td>CL3360</td>
<td>mII872::Kan’</td>
<td>SR108 × P1 (JW2040)</td>
</tr>
<tr>
<td>CL3362</td>
<td>mIIA733::Kan’</td>
<td>SR108 × P1 (CL1406)</td>
</tr>
<tr>
<td>CL1420</td>
<td>katE::FRT-cat-FRT</td>
<td>CL1424 × pCP20 (eliminate cat)</td>
</tr>
<tr>
<td>CL1424</td>
<td>katE::FRT</td>
<td>CL1424 × P1 (CL1408)</td>
</tr>
<tr>
<td>CL1429</td>
<td>katE::FRT katG::FRT-cat-FRT</td>
<td>CL1429 × P1 (CL1408)</td>
</tr>
<tr>
<td>CL1438</td>
<td>katE::FRT katG::FRT</td>
<td>CL1438 × P1 (CL1408)</td>
</tr>
<tr>
<td>CL1440</td>
<td>katE::FRT katG::FRT recA::cat</td>
<td>CL1440 × P1 (CL1408)</td>
</tr>
</tbody>
</table>
For all treatments, cultures were immediately returned to 37°C following exposure to allow continued growth and recovery in the case of UV-irradiated and hydrogen peroxide-treated cells. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [3H]thymidine for 2 min at 37°C. The cells were then lysed, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass filters. The amounts of [3H] and [14C] on each filter were determined by scintillation counting.

To determine whether the recovery of synthesis involves extensive rNTP incorporation, cultures were grown and treated with freshly prepared 100 mM hydroxyurea as described above. At the times indicated, quadruplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [3H]thymidine for 2 min at 37°C. Cells from two of the four aliquots were then lysed immediately, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters as before. The remaining two aliquots were lysed and treated with alkali at a final concentration of 500 mM NaOH for 30 min at room temperature before the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of [3H] on each filter were determined by scintillation counting.

To determine whether recovery of synthesis requires transcription or new protein synthesis, cultures were grown as described above and then divided into six aliquots. Three of the six aliquots were mock treated together with no inhibitor, 100 μg/ml rifampin (transcription inhibitor), or 150 μg/ml chloramphenicol (translation inhibitor). The remaining three aliquots were treated with freshly prepared 100 mM hydroxyurea together with no inhibitor, 100 μg/ml rifampin, or 150 μg/ml chloramphenicol. Samples were collected at the times indicated, pulse-labeled with 0.5 μCi/ml [3H]thymidine for 2 min at 37°C, and processed as described above.

RNA synthesis. Overnight cultures were diluted 1:100 and grown at 37°C in DGCthy to an OD600 of precisely 0.3, at which point half of the cells were mock treated, while the remaining culture was treated with freshly prepared 200 mM hydroxyurea. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.2 μCi/ml [3H]uridine for 2 min at 37°C. The cells were then lysed, and the RNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amount of [3H] on each filter was determined by scintillation counting.

Genomic DNA preparation and agarose gel analysis. Fresh overnight cultures were diluted 1:100 in DGCthy medium, grown at 37°C to an OD600 of 0.3, treated with freshly prepared 100 mM hydroxyurea, and then incubated further at 37°C. At the times indicated, a 0.75-ml aliquot of culture was transferred to an equal volume of NET (100 mM NaCl, 10 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0]), centrifuged for 60 s, resuspended in 140 μl of lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris [pH 8.0], 0.5 mM EDTA [pH 8.0], 1 mM EDTA [pH 8.0]), and incubated at 37°C for 30 min. Ten microliters of 10 mg/ml proteinase K and 10 μl 20% Sarkosyl were then added, and incubation at 37°C was performed for a further 30 min. Samples were then extracted with four volumes of phenol-chloroform (1:1), followed by four volumes of chloroform.

Samples were treated with 200 mM NaOH for 30 min and then electrophoresed on a 0.5% alkali-agarose gel in 30 mM NaOH, 1 mM EDTA at 30 V for 16 h, stained, and visualized with ethidium bromide. A second set of samples were electrophoresed on a 0.5% neutral-agarose gel in 30 mM NaOH, 1 mM EDTA at 30 V for 16 h, stained, and visualized with ethidium bromide.

Measuring hydroxyurea survival in liquid cultures. Fresh overnight cultures were diluted 1:100 in DGCthy medium, grown at 37°C to an OD600 of 0.3, and then treated with 100 mM hydroxyurea that was either freshly prepared or previously heat treated for 48 h at 37°C as described above. At the times indicated, 0.1-ml aliquots of each culture were removed and serially diluted in DGCthy medium in 10-fold increments. Triplicate 10-μl aliquots of each dilution were then spotted on Lbthy plates. Viable colonies were counted following overnight incubation at 37°C.

Measuring hydroxyurea survival on agar plates. Fresh overnight cultures were diluted 1:100 in DGCthy medium and grown at 37°C to an OD600 of 0.3. Triplicate 10-μl aliquots of serial 10-fold dilutions were then spotted on Lbthy plates containing hydroxyurea at the indicated doses. Viable colonies were counted following overnight incubation at 37°C.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00713-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES


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Figure S1. *Transcription is inhibited in the presence of hydroxyurea.* [³H]-uridine was added to cultures for 2 min at the indicated times following treatment with hydroxyurea at time zero. The rate of RNA synthesis, as monitored by [³H]-incorporation, relative to the rate immediately prior to treatment is plotted for wild-type cells over time. Mock-treated (open squares), 200 mM hydroxyurea (filled squares).
Figure S2. Free radical damage is not responsible for the initial inhibition of replication that occurs when fresh hydroxyurea is added to cultures. A) Hydroxyurea-mediated, free radical DNA damage requires catalase. The survival of wild-type, recA, katEkatG, and katEkatGrecA mutants on agar plates supplemented with 10 mM hydroxyurea is shown. 0.01 ml drops of 10-fold serial dilutions were spotted on agar plates containing 10 mM hydroxyurea. B) The presence or absence of catalase, which is required to form free radical DNA damage, does not affect the inhibition or recovery of replication when fresh hydroxyurea is used. The observation argues that the initial inhibition is not caused by free radical damage. The rate of synthesis and DNA accumulation were measured as in Figure 1 for katEkatG and katEkatGrecA mutants. 100 mM hydroxyurea (filled symbols); mock treatment (open symbols). total DNA accumulation (14C; circles); rate of DNA synthesis (3H; squares). Graphs represent an average of two independent experiments. Error bars represent one standard error of the mean.
Figure S3. DNA synthesis in the presence of hydroxyurea initially resumes, but does not fully recover, in the absence of transcription or protein synthesis. The rate of synthesis and DNA accumulation in wildtype cultures were measured as in Figure 1. 100 µg/ml rifampicin, 150 µg/ml chloramphenicol was added to cultures immediately prior to hydroxyurea treatment. 100 mM hydroxyurea (filled symbols); mock treatment (open symbols). total DNA accumulation (\(^{14}C\); circles); rate of DNA synthesis (\(^{3}H\); squares).