Characterization of the Replication Rate and Intermediates Produced Following Hydroxyurea treatment in *Escherichia coli*

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Abstract

In order to reproduce, all cells must duplicate their genomes accurately. Active replication forks encounter impediments such as DNA adducts, strand breaks, bound proteins, or secondary DNA structures that impair their ability to duplicate the DNA. UV-irradiation causes DNA damage that arrests replication forks and induces distinct intermediates during the recovery process. However, less is known about how other impediments to replication are processed. Hydroxyurea is thought to stall replication and induce oxidative DNA damage by inhibiting ribonucleotide reductase. Here I characterize how replication responds to treatment with hydroxyurea and identify the replication intermediates that arise in *Escherichia coli*. I show that replication is initially inhibited by treatment with 0.1 M hydroxyurea. However following inhibition, replication unexpectedly recovers for a period of time. Two-dimensional (2D) agarose gel analysis of the replicating plasmid pBR322 indicated that fork regression and processing intermediates arise at the stalled replication forks similar to UV-induced DNA damage. The transient recovery of DNA synthesis was found to not be due to upregulation of the cryptic ribonucleotide reductase, NrdEF. These results suggest that cells possess a means to temporarily synthesize DNA in the presence of low dNTP concentrations using a mechanism that shares similar intermediates with those arising following UV-induced damage.
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1) Introduction

1.1 Replication after DNA damage

In order to reproduce successfully, all cells must duplicate their genomes accurately (reviewed in 1, 2). As the cell duplicates its genome it encounters a variety of impediments to replication (1). Environmental and chemical factors around the cell can produce structural changes that can damage the DNA molecule (1). Heat, chemicals, and certain wavelengths of irradiation can result in DNA adducts, strand breaks, bound proteins, or DNA secondary structures (1). If left unchecked, these impediments can result in undesirable consequences such as DNA mutation or cell lethality in the affected cell or its progeny (1). Chemical changes such as DNA lesions can either block replication completely in the affected cell preventing it from multiplying, or they can introduce mutations or rearrangements into its progeny which may be deleterious or lethal (1). Since the maintenance of individual survival (and by extension continued propagation of the species) depends upon some degree of genomic stability (2), cells have evolved mechanisms to repair this damage as they replicate their genomes (1).

1.2 Replication after UV-irradiation induced DNA damage

The most studied examples of impediments to replication are the DNA lesions caused by UV-irradiation (3, reviewed in 1). UV induced damage produces bulky lesions such as cyclobutane pyrimidine dimers and 4-6 photoproducts which prevent the replication machinery from progressing and completing duplication of the genome (4). The primary mechanism for removing UV-induced lesions from the genome is nucleotide excision repair which requires UvrA, UvrB, UvrC, and UvrD along with Pol I and ligase (reviewed in 5). If cellular replication machinery encounters the lesion prior to repair occurring, then the activity of several RecF pathway proteins (RecA, RecF, RecO, RecR, RecQ, and RecJ) becomes critical for survival (1).

After replication fork arrest by UV induced damage, the RecJ nuclease and RecQ helicase partially unwind and degrade the nascent lagging strand (6). In recQ or recJ mutants, it was observed that the nascent strand does not degrade (6). RecF, RecO, and RecR limit this degradation and recruit RecA to protect and maintain the DNA strands of the replication fork (7, 8). Together, these four proteins promote a transient regression of the replication fork (7, 8). It is thought that this regression allows repair enzymes or a translesion DNA polymerase to access the
lesion and enable replication to overcome it by either repairing the damage or bypassing it (8). In cells lacking these proteins, replication fails to recover and the DNA at the stalled fork is rapidly degraded (7, 8). Once replication overcomes the lesion, it is thought that replication machinery reassembles and replication resumes (9).

1.3 Observing replication fork regression in vivo with two dimensional (2D) gel electrophoresis

The transient regression of the replication fork induced by arresting lesions can be observed in vivo, using two-dimensional (2D) gel electrophoresis on plasmids such as pBR322 (8). On 2D gels, normal replicating DNA molecules migrate through the gel as “Y” shaped structures and appear as an arc on the gel (see Fig. 4 A). Processing intermediates induced by the damage however, migrate as “X” shaped molecules and appear as a distinct cone region on the gel (Fig. 4 A). Consistent with the proposed repair model previously discussed, these “X” shaped intermediates accumulate in wild-type cells following 50J/m² of UV irradiation and then begin to resolve within 30 minutes after irradiation, before disappearing altogether by 60 minutes (8). The time it takes the intermediates to disappear was found to correlate with the time it takes for repair to be completed and DNA synthesis to resume (8). Mutants of uvrA, which cannot remove the block to replication (10), failed to recover and X- shaped intermediates persist and accumulate over time without disappearing (8). In recA, recF, recO, or recR mutants, where regression does not occur and the nascent DNA is degraded, replication also fails to recover, but intermediates are not observed (8, 11). The appearance of replication intermediates could be partially restored in recF, recO and recR mutants by inactivating RecJ or RecQ which degrade DNA at the blocked fork (8, 11).

The use of 2D agarose gel electrophoresis can also reveal other intermediates that are not directly associated with restoring DNA synthesis but have roles in maintaining genome stability. Mutants such as ruvAB or ruvC (which lack a functional enzyme complex for resolution of Holliday junctions and branch migration (12, 13)) are hypersensitive to UV-induced damage but remain proficient in restoring replication after DNA arrest (14). These mutants experience lethality several hours after recovering replication, because accumulated Holliday junctions in the replicated DNA fail to resolve (15). On a 2D agarose gel analysis, both ruvAB ad ruvC mutants show a distinct complex of higher order intermediate multimer plasmids after UV-
damage (seen as secondary or tertiary cone regions above the main cone region) that contain unresolved Holliday junctions (15).

Thus, 2D agarose gel analysis is a useful technique for identifying both the transient regression of the replication fork and other more complex intermediates associated with maintaining genomic stability. This technique can therefore be used to reveal the defects in DNA processing in mutants of specific processing related genes, through the display of intermediates that accumulate (or fail to accumulate) in the absence of those functional genes.

1.4 Replication during Hydroxyurea induced stalling

The cell’s response to UV-induced DNA damage has been well characterized; however, much less is known about how replication responds to other forms of impediments.

Hydroxyurea (HU) is an important chemical agent that has been used as a chemotherapeutic (16, 17), antiviral (18), and treatment for sickle cell anemia (reviewed in 19). Its potential medical applications (both alone and in combination with other drugs) have been extensively explored since the 1960s.

Hydroxyurea is thought to stall replication by inhibiting ribonucleotide reductase. Once it enters the cell, it is converted into a free radical nitroxide, which scavenges the tyrosyl free radical from the active site of the M2 protein subunit of class I ribonucleotide reductases (RNRs) (20, 21). Without these free radicals, the M2 subunit of the RNR complex is not able to catalyze the reduction of ribonucleotide diphosphates (NDPs) to their respective deoxyribonucleotides (dNDPs) (22). Ribonucleotide reductases accomplish this through the following two step reaction sequence (22):

1. \[ \text{NADPH} + \text{H}^+ + \text{thioredoxin-S}_2 \rightarrow \text{thioredoxin-(SH)}_2 + \text{NADP}^+ \]
2. \[ \text{thioredoxin-(SH)}_2 + \text{NDP} \rightarrow \text{thioredoxin-S}_2 + \text{dNDP} \]

As the cell’s RNRs become inactivated, the entire replicase complex for producing and delivering dNTPs (of which the RNR is a smaller part (23)) shuts down (20). Cells then become starved for the deoxyribonucleotide triphosphates required for DNA synthesis and replication stalls (24).

In addition to this mechanism of action, recent studies suggest that hydroxyurea indirectly generates oxidative DNA damage in the cell (25). When the replication fork stalls as a response to nucleotide starvation, downstream of the stalled forks \textit{mazEF} and \textit{relBE} toxin-antitoxin pairs
are activated; *mazE* and *relB* toxin suppressors are deactivated and *mazF* and *relE* growth inhibiting toxins lead to protein misfolding and membrane stress (26). This in turn can alter the properties of one of the cytochrome oxidases in the membrane-associated electron transfer chain (ETC), releasing superoxide and hydroxyl radicals that can damage membranes, proteins and DNA (26). In response to RNR depletion the cell also increases its uptake of iron (in an attempt to restore activity to its RNRs); however this excess iron catalyzes the production of more superoxide radicals, aggravating the damage produced through the toxin-antitoxin pathway (26).

Several studies have identified genes that are important for survival following hydroxyurea treatment. In *E. coli*, the *umuC, umuD, umuD’*, and *dinB* genes (which encode for the DNA polymerases Pol IV and Pol V) play a role in survival after hydroxyurea treatment (27). It has been found that Pol IV and Pol V mutants are more resistant to hydroxyurea than wild type cells (27). Although the mechanism for this remains unclear, it is possible that the absence of Pol IV and Pol V prevents DNA synthesis from resuming when nucleotide pools are low or imbalanced, which could be lead to toxic levels of damaged or misincorporated bases in the DNA. Additionally, *recA* mutants are hypersensitive to hydroxyurea (28) and the SOS response is known to be induced following treatment (29 reviewed in 26). In budding yeast 2D gel electrophoresis analysis of DNA stalled with hydroxyurea has indicated that Mec1 and Rad53 proteins play a role in promoting cell survival (30).

1.5 Characterizing replication intermediates formed during Hydroxyurea induced stalling

While a number of genes have been identified to play a role in hydroxyurea resistance, how replication responds to hydroxyurea induced stalling in vivo has not yet been examined. Here, I characterized the effect of different concentrations of hydroxyurea on the rate of DNA synthesis, and identified the replication intermediates on DNA molecules that arise in the presence of this drug in *Escherichia coli (E. coli)*. I accomplished this using replication inhibition assays to track the rate of synthesis, and 2D agarose gel electrophoresis to distinguish the replication intermediates.
2) Materials and Methods

2.1 Bacterial Strains, Plasmids, and Culture Medium

SR108 is a thymine auxotroph (thyA36 deoC2) derivative of W3110 (31). The plasmid pBR322 is a medium copy number, ColE1-based, 4.4-kb plasmid with ampicillin resistance (Promega). MG1655 and MG1655 nrdEF are thymine prototrophs, derivatives of W1485 (32), and have been described previously (33). The nrdEF gene encodes the aerobic, manganese-dependant, class Ib, ribonucleotide reductase. All bacterial strains were propagated in Davis medium supplemented with 0.4% glucose and 0.2% cas amino acids (DGC). DGC growth medium for thymine auxotrophs was also supplemented with 10 µg/ml thymine (DGCth). 

2.2 Replication Inhibition Assays

For experiments using thymine auxotrophs, overnight cultures were diluted 1:100 into DGC medium supplemented with 10 µg/ml thymine and 0.1 µCi/ml $^{14}$C-thymine (Moravek Biochemicals) and grown at 37°C in a shaking water bath to an optical density at 600 nm (OD$_{600}$) of precisely 0.3, at which point the culture was divided equally into four flasks and treated with 0, 1, 10 or 100 mM hydroxyurea (MP Biomedicals). For control experiments using UV irradiation as the DNA damaging agent, the main culture was divided equally and either irradiated at an incident dose of 30J/m$^2$ under a 15-watt germicidal lamp (254 nm), or mock irradiated.

For experiments using thymine prototrophs, overnight cultures were diluted 1:100 into DGC medium and grown at 37°C in a shaking water bath to an OD$_{600}$ of precisely 0.3, at which point each culture was divided equally into two flasks and treated with 0 or 100 mM hydroxyurea.

For both hydroxyurea treatment and UV irradiation, cultures were returned immediately to 37°C following exposure to allow recovery and continued growth.

At the indicated time points, duplicate 500 µl aliquots of culture were removed from the treated flasks and pulsed with 1 µCi/ml $^3$H-thymidine (Moravek Biochemicals) for 2 min at 37°C. Cells were then lysed with 5% cold trichloroacetic acid (TCA, Fisherbrand) and the precipitated DNA was collected on glass fiber filters (Millipore). The filters were rinsed twice with ethanol, dried, and then the amount of $^3$H and $^{14}$C on each filter was determined using a scintillation counter.
2.3 Two-Dimensional (2D) Agarose Gel Electrophoresis

Cultures of SR108 containing the plasmid pBR322 were grown overnight in DGCthy medium supplemented with 100μg/ml ampicillin. 1-ml aliquots of this overnight culture were pelleted out and resuspended in 1ml of DGCthy medium without ampicillin. 200μl was then used to inoculate 20ml of DGCthy medium without ampicillin and allowed to grow at 37°C until cells reached a density of 5x10^8 cells/ml. At this point cultures were either treated with 100 mM hydroxyurea or UV irradiated with 50J/m^2 and transferred to a fresh pre-warmed flask. At the indicated time points 750-μl aliquots of culture were removed from the treated flasks and placed into an equal volume of cold NET (100 mM NaCl, 20 mM EDTA, pH 8.0, 10 mM Tris, pH 8.0). The cells in each sample were then pelleted, resuspended in 150-μl lysis buffer (1.5 mg/ml lysozyme, 0.5 mg/ml RNaseA in 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0), and incubated for 30 min at 37°C. Then, 10 μl of 10 mg/ml proteinase K and 10 μl of 20% sarkosyl were added to each sample, and incubated for a further 30 min at 37°C. Samples were then extracted once in four volumes of phenol/chloroform, once in four volumes of chloroform, dialysed against 200-ml of TE (2 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min on floating 0.025-μM dialysis membranes (Millipore), and digested overnight with PvuII (Fermentas) at 37°C.

Samples were extracted with one volume of chloroform, and equal volumes of sample were then loaded onto a 0.4% agarose gel in 1X TBE (Tris-borate-EDTA, pH 8.0). DNA was initially separated in this first dimension at 1V/cm for 20 hours. For the second dimension, gel lanes were cut out, rotated 90°, recast in a 1.0% agarose gel in 1X TBE, and electrophoresed for 6.5 h at 6.5 V/cm. DNA in the gel was transferred to HybondN+ nylon membranes (GE Healthcare) as previously described (4, 15), and the plasmid DNA was detected using pBR322 DNA that had been labeled with alpha[^32]P-dCTP using nick translation (Roche), and visualized using a Storm 820 PhosphorImager and its associated ImageQuant Software (GE LifeSciences).
3) Results

3.1 UV irradiation temporarily inhibits replication in wild type cultures

Previous work has found that after exposure to 27J/m² of UV irradiation in wild type cultures the rate of DNA synthesis is initially inhibited by approximately 90% before the rate recovers to near pre-treatment levels within 60 minutes post irradiation (8, 16). For the purpose of controls, this experiment was repeated and is shown in Fig. 1 A and B.

![Graph A](image)

**Fig. 1**
Rate of DNA synthesis and total DNA accumulation over time after exposure to UV irradiation. Cultures grown with $^{14}$C-thymine were labeled with a pulse of $^3$H-thymidine for 2 min, after either 30J/m² of UV-irradiation or mock irradiation. The amounts of $^3$H and $^{14}$C incorporated into the DNA (relative to pre treatment levels) were measured and are plotted over time. Both graphs presented represent data from one experiment. (A) Open squares- relative [$^3$H] incorporation/ 2 min in mock irradiated cultures. Closed circles- relative [$^3$H] DNA incorporation/ 2 min in UV-irradiated cultures. (B) Open squares- relative [$^{14}$C] DNA accumulation in mock irradiated cultures. Closed circles- relative [$^{14}$C] DNA accumulation in UV-irradiated cultures. $^3$H cpm and $^{14}$C cpm at -5 minutes were: 13022.20 and 2805.67 respectively.

The rate of DNA synthesis after 30J/m² of UV-irradiation was tracked by measuring the amount of $^3$H-thymidine incorporated during a 2 min pulse into DNA pre-labeled with $^{14}$C-thymine.

Consistent with results previously obtained (14, 15), it was found that after UV-irradiation the rate of DNA synthesis was temporary inhibited by approximately 90% (Fig.1 A). After approximately 20 min, replication rates began to recover nearly reaching pre-treatment levels by
the end of the 60 minute time course (Fig.1 A). Similarly, though total DNA accumulation was reduced after irradiation, the DNA began to accumulate again by the end of the time course (Fig. 1 B). As seen in Fig. 1 B, untreated cultures accumulated approximately 75% more total DNA than treated cultures by the end of the 60 min time course.

3.2 *At a concentration of 100 mM, hydroxyurea temporarily inhibits replication in wild type cultures.*

In order to examine what effect hydroxyurea has on DNA replication, we began by repeating the experiment above. However, instead of UV irradiating the cultures, various concentrations of hydroxyurea were added at the start of the experiment to serve as the replication fork stalling agent (Fig. 2 A and B).

The rates of DNA synthesis, after treatments of 0mM, 1mM, 10mM, and 100mM concentrations of hydroxyurea, were followed by measuring the amount of $^3$H-thymidine incorporated during a 2 min pulse into cultures pre-labeled with $^{14}$C-thymine. The results are presented in Fig. 2 A and B.

The extent to which DNA synthesis was initially inhibited was found to depend on the concentration of hydroxyurea added. Treatments of 1mM hydroxyurea did not show a significant drop in the rate of DNA synthesis compared to the untreated control. Treatments of 10mM hydroxyurea initially inhibited DNA synthesis by approximately 70% before replication began to recover around 10 min after treatment and return to pre treatment rates after about 20min (Fig. 2 A). Hydroxyurea treatments of 100mM initially inhibited synthesis by 90%-95%. After approximately 10 min post-treatment, cultures began to recover replication and return to pre treatment rates by 50 min (Fig. 2 A).

Total DNA accumulation was also found to be dependant on the concentration of hydroxyurea. The 1mM HU treatment had no significant effect on the total DNA produced after treatment, while after 10mM HU and 100mM HU treatments the total DNA produced was significantly less (Fig. 2 B). As seen in Fig. 2 B, 10mM and 100mM HU treated cultures accumulated about 36% and 49% less total DNA respectively than untreated cultures by the end of the 60 min time course.
Fig. 2
Rate of DNA synthesis and total DNA accumulation over time after addition of 0mM, 1mM, 10mM, and 100mM hydroxyurea to growing _E. coli_ cultures. Cultures grown with ^14^C-thymine were labeled with a pulse of ^3^H-thymidine for 2 min at various time points following the addition of hydroxyurea. The amounts of ^3^H and ^14^C incorporated into the DNA (relative to pre-treatment levels) are plotted over time. Graphs represent data from an average of 4 independent experiments with error bars representing one standard deviation. (A) [^3^H] DNA incorporated/ 2 min in: open squares - 0mM HU; closed circles - 1mM HU; closed triangles - 10mM HU; closed diamonds -100mM HU. (B) Total [^14^C] DNA accumulation in: open squares - 0mM HU; closed circles - 1mM HU; closed triangles- 10mM HU; closed diamonds - 100mM HU. Average ^3^H cpm and ^14^C cpm at -5 minutes were: 10981.04 and 2730.62 respectively.

The recovery of DNA synthesis observed in the presence of hydroxyurea was unexpected. In theory, in the presence of this drug, pools of dNTPs would be expected to remain depleted, and no recovery should occur. Furthermore, extended incubation of cells in 100mM hydroxyurea has been shown to inhibit further growth and reduce cell viability (26). Thus we hypothesize that the cell must have some mechanism in place to allow replication to resume at least temporarily after dNTP depletion. This could be by either restoring dNTP levels to acceptable amounts, or by allowing synthesis to occur despite low levels of dNTP precursors.
3.3 E. coli lacking the nrdEF ribonucleotide reductase also recover replication to pre-treatment levels following damage with hydroxyurea.

E. coli contains a cryptic Mn-dependant ribonucleotide reductase, encoded by the nrdEF operon (35). NrdEF is considered a cryptic enzyme because its specific function in the cell is still unknown. We hypothesized that this cryptic enzyme is induced in the presence of hydroxyurea and transiently restores dNTP concentrations to a level that allows the recovery of DNA synthesis. In order to examine this possibility, I compared the ability of wild type and nrdEF mutants to recover DNA synthesis following the addition of hydroxyurea by repeating the experiment above. The results of this assay are presented in Fig. 3.

![Graph showing rate of DNA synthesis over time in wild type and nrdEF mutants after addition of 100mM hydroxyurea.](image)

**Fig. 3**
Rate of DNA synthesis over time in wild type and nrdEF mutants after addition of 100mM hydroxyurea. Cultures were labeled with a pulse of $^{3}$H-thymidine for 2 min, after treatment with 100mM HU. The amounts of $^{3}$H incorporated into the DNA (relative to pre treatment levels) are plotted over time. Graphs represent data from an average of 2 independent experiments with error bars representing one standard deviation. Open squares show $[^3]$H DNA incorporated/2 min in untreated wild type cultures while closed circles show $[^3]$H DNA incorporation/2 min in 100mM HU treated wild type cultures. Open triangles show $[^3]$H DNA incorporation/2 min in untreated nrdEF mutants while closed diamonds show $[^3]$H DNA incorporation/2 min in 100mM HU treated nrdEF mutants. Since the wild type strain used in this assay (MG1655) is thyA+, and is therefore unable to take up $^{14}$C thymine, total DNA accumulation was not measured and only the rate of DNA synthesis was monitored. $^3$H cpm at -5 minutes was: 24657.5 for wild type cultures, and 23271.5 for nrdEF mutants.

The rate of DNA synthesis after HU treatment was found to recover similarly in both the wild-type control and the nrdEF mutant. In both cases, treatments of 100mM HU initially inhibited DNA synthesis by approximately 90% before replication began to recover around
10min after treatment and return to nearly pre treatment rates after about 50min (Fig. 3). We interpret these results to indicate that the ability of cells to temporarily recover DNA synthesis in the presence of hydroxyurea is not due to the action of the cryptic ribonucleotide reductase, NrdEF.

From these assays it was determined that 100mM concentrations of hydroxyurea were enough to produce a temporary inhibition in DNA synthesis. This concentration of HU was subsequently used to examine potential replication intermediates in the 2D gel electrophoresis analysis.

3.4 DNA damaged by hydroxyurea produces replication intermediates similar to those formed after UV irradiation.

Previous work has found that after a dose of 50J/m² of UV irradiation, DNA on the pBR322 plasmid generates X-shaped replication intermediates that persist for 30 min until the UV-induced lesions are removed and cells can resume replication (7). In order to examine the intermediates that occur after treatment with replication inhibiting (100mM) concentrations of hydroxyurea, a 2D agarose gel electrophoresis analysis was undertaken.

This technique was chosen because it is able to separate and identify the structures of replicating DNA fragments (Fig. 4 A). After cultures are treated with a DNA damaging agent (UV-irradiation or HU), the total genomic DNA is purified from the culture at various times after treatment and the pBR322 plasmid DNA is isolated and linearized just downstream of its replication origin using the PvuII restriction enzyme, before analysis by 2D gel electrophoresis.

Nonreplicating linearized plasmids migrate as 4.4kb fragments, while normal replicating fragments show up as an arc extending from the linear fragment spot, due to their bulkier Y-shape and larger sizes (4, 15). X-shaped replication intermediates show up in a cone region above the arc because their more complex shapes migrate even slower than that of normal replicating molecules (4, 15). Higher order branched intermediates show up as secondary or tertiary cone regions above the main cone region. Plasmids that contain gaps (and as a consequence may be resistant to digestion by PvuII) show as smaller spots above the 4.4kb fragment (4, 15) due to their size and circular shape.
Fig. 4
DNA replication intermediates observed after damage with hydroxyurea and UV irradiation. (A) PvuII digested pBR322 follows the migration pattern shown when observed by 2D agarose gel electrophoresis. Nonreplicating linearized plasmids run as 4.4kb fragments and show up as a large round spot. DNA fragments that are replicating normally form Y shaped structures and show up as the arc extending from the linear fragment spot. X-shaped replication intermediates run in the cone region. Any unrestricted circular plasmids that contain gaps show as smaller spots above the 4.4kb fragment. (B) 2D agarose gels of pBR322 DNA after 50J/m² of UV irradiation (C) 2D agarose gels of pBR322 DNA after treatment with 100mM HU.
For the purposes of a control, 2D gel electrophoresis analysis using 50J/m² of UV irradiation as the DNA damaging agent was performed (Fig. 4 B and D). Consistent with previously obtained results (4, 15), after damage, blocked replication forks and cone region replication intermediates were observed to accumulate in samples prepared 15 and 30 min post irradiation. Furthermore, these intermediates disappeared by 90 min (data not shown).

When the 2D agarose gel electrophoreses analysis was performed using cultures treated with 100mM HU, we observed similar blocked replication forks and accumulation of cone region replication intermediates (Fig 3 C and E).

Although it would be necessary to repeat these experiments, the results suggest that hydroxyurea treatment induces replication fork processing and intermediates similar to those seen after arrest by UV-induced damage.

4) Discussion

In this study I characterized the effect of hydroxyurea on DNA synthesis in vivo. Unexpectedly, I observed that DNA synthesis temporarily resumed, even in the presence of lethal concentrations of hydroxyurea. Recovery was not dependent on the cryptic ribonucleotide reductase NrdEF. This suggests that in E. coli, replication can resume despite reduced concentrations of dNTPs following hydroxyurea treatment.

It would be of interest to see which DNA polymerases are capable of carrying out synthesis under these conditions. It has been shown that Pol IV and Pol V mutants are less sensitive to killing by hydroxyurea (27), suggesting that the presence of these two polymerases plays a role in contributing to the lethal effects of hydroxyurea. To further investigate their role in post treatment DNA synthesis, one could look at whether synthesis in mutants for these polymerases fails to recover in the presence of HU. If Pol IV and Pol V contribute to hydroxyurea survival, then the recovery of synthesis that occurs after HU addition to the media should be prevented or impaired in mutants lacking these polymerases.

This study has provided evidence that treatment with HU results in the production of branched replication intermediates in the cell that resemble those formed by UV-induced damage. If these intermediates were identical to those generated by UV-induced damage, then their formation should be catalyzed by RecA and RecF, RecO and RecR (7). Whether the same
proteins are involved in maintaining these intermediates formed after HU inhibition remains to be answered. Thus it would be interesting to examine how mutants lacking these recombinase genes react to HU damage. One prediction is that HU damage induced intermediates would fail to form in these mutants similar to what happens following UV damage.

The HU-induced replication intermediates that were observed might indicate that fork stalling alone is sufficient to induce the processing events, even in the absence of a DNA lesion. Alternatively, replication forks might be encountering oxidative DNA damage and the resulting lesions that are thought to be produced as a secondary effect of HU treatment (25). These oxidative lesions might then induce processing in a similar manner to UV induced lesions. However, the latter possibility seems less likely considering that damage induced processing intermediates have not been observed after oxidative damage to the DNA. Even strong oxidative challenges, such as treatment with 10mM hydrogen peroxide, have not been observed to produce processing intermediates (Brandy Schalow, personal communication). To differentiate between fork stall and oxidative damage as the cause of these intermediates, it might be informative to see whether the effects of other forms of replication stalling such as thymine starvation (35) produce similar processing of the replication forks. If intermediates are seen after thymine starvation (which does not induce oxidative damage) then it would suggest that fork stalling alone is sufficient to induce the processing events.

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References


