

Replication fork stalling and cell cycle arrest in UV-irradiated *Escherichia coli*

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Faithful duplication of the genome relies on the ability to cope with an imperfect template. We investigated replication of UV-damaged DNA in *Escherichia coli* and found that ongoing replication stops for at least 15–20 min before resuming. Undamaged origins of replication (*oriC*) continue to fire at the normal rate and in a DnaA-dependent manner. UV irradiation also induces substantial DnaA-independent replication. These two factors add substantially to the DNA synthesis detected after irradiation and together mask the delay in the progression of pre-existing forks in assays measuring net synthesis. All DNA synthesis after UV depends on DnaC, implying that replication restart of blocked forks requires DnaB loading and possibly the entire assembly of new replisomes. Restart appears to occur synchronously when most lesions have been removed. This raises the possibility that restart and lesion removal are coupled. Both restart and cell division suffer long delays if lesion removal is prevented, but restart can occur. Our data fit well with models invoking the stalling of replication forks and their extensive processing before replication can restart. Delayed restart avoids the dangers of excessive recombination that might result if forks skipped over lesion after lesion, leaving many gaps in their wake.

[**Keywords:** Fluorescent microscopy; BrdU labelling; excision repair; DnaA; DnaB; DnaC]

Supplemental material is available at <http://www.genesdev.org>.

Received November 8, 2006; revised version accepted January 29, 2007.

At the root of genomic instability lies the raw fact that evolution is concerned with survival rather than with exact transmission of the genome. Organisms survive and reproduce because they exploit repair systems to reduce or eliminate lesions from the DNA and use surveillance mechanisms (checkpoints) to make sure cells progress through the cell cycle only when it is appropriate to do so. Thus, the G₁-S transition checkpoint inhibits initiation of DNA replication in eukaryotes if there are lesions in the template. The delay provides time for repair activities to restore the template, after which replication might proceed unhindered. Without such coordination, there is increased risk of mutation, genomic instability, and cell death (Myung et al. 2001).

There appears to be no G₁-S checkpoint in bacteria. In an early model based on studies of the DNA synthesized in UV-irradiated *Escherichia coli* cells, Rupp and Howard-Flanders (1968) proposed that replication forks simply proceed past the damage and resume synthesis downstream, leaving gaps that are then filled in by RecA-mediated recombination (Fig. 1A, i). They estimated that replication is delayed no more than ~10 sec per lesion. This post-replication repair model had one major drawback. It was held almost as dogma that the priming of

leading strand synthesis is restricted to *oriC* (Courcelle and Hanawalt 2003). So, how could replication resume downstream from a lesion? A possible solution emerged recently. Heller and Marians (2006) showed that synthesis of the leading strand could be initiated de novo at fork structures, at least in vitro, raising the possibility that leading strand synthesis could in fact restart downstream from a lesion.

In the intervening period, Meneghini and Hanawalt (1975) suggested that a lesion in the leading strand template blocks fork progression whereas a lesion in the lagging strand template does not. The lagging strand polymerase simply skips an Okazaki fragment, leaving a gap. This model has been strongly supported by in vitro as well as in vivo data showing that lesions in the template can indeed disrupt the coupled synthesis of leading and lagging strands (Higuchi et al. 2003; Pages and Fuchs 2003; McInerney and O'Donnell 2004). In vivo, replication of the leading strand was delayed for a substantial period (Pages and Fuchs 2003). This delay conflicts with the Rupp and Howard-Flanders model but fits with data showing that the rate of DNA synthesis drops dramatically immediately after UV irradiation (Khidhir et al. 1985; Courcelle et al. 2005, 2006). Synthesis recovers in wild-type cells but not in excision repair-defective *uvrA* mutants, except at low UV doses, indicating that nucleotide excision repair is important for recovery.

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.417607>.

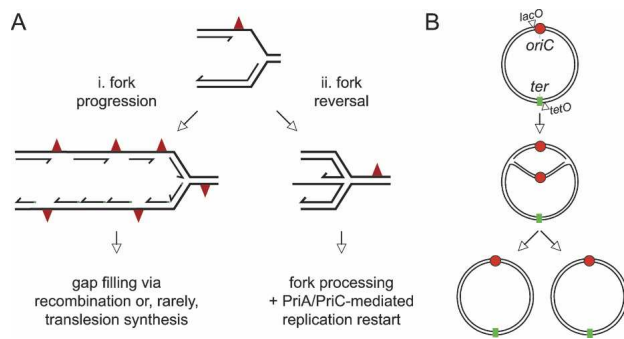


Figure 1. DNA replication and replication restart after UV irradiation in *E. coli*. (A) Models of replication restart: (i) A fork skips over lesions (red triangles) leaving gaps in the nascent strands. (ii) A fork stalls at a leading strand block and reverses to form a Holliday-junction structure that is then processed to allow restart. (B) Diagram illustrating initiation and termination of chromosome replication. The open triangles indicate the positions of the *lacO240* and *tetO* arrays used.

But how does replication resume if the lesion is masked by the stalled replisome or cannot be repaired because the template is unwound? Higgins et al. (1976) and Fujiwara and Tatsumi (1976) presented evidence that blocked forks reverse in mammalian cells to form a Holliday-junction structure, which can then be exploited to restart replication (Fig. 1A, ii). In recent years, the idea that extensive fork processing might be associated with replication restart has gained momentum (McGlynn and Lloyd 2002; Michel et al. 2004). It stands in sharp contrast to the original model of Rupp and Howard-Flanders (1968).

In this study, we provide evidence that lesions induced by UV delay ongoing replication quite markedly and that initiation of new rounds of replication together with the triggering of UV-induced synthesis can mask this delay. Our data also show that ongoing replication recovers eventually and that this recovery depends on loading of the replicative helicase and, to a large extent, on lesion removal while replication is delayed.

Results

Replication in *E. coli* initiates when DnaA protein binds the single origin of replication (*oriC*), opens the duplex, and facilitates transfer of DnaB helicase from a DnaB:DnaC complex to each of the template strands. This leads to the assembly of two replisomes, which then move in opposite directions around the chromosome (Marians 1992). Duplication of the entire chromosome is achieved when two forks meet at the terminus (*ter*) (Fig. 1B). The cell cycle is completed when the chromosomes segregate and division occurs (Lau et al. 2003; Sherratt 2003; Wang et al. 2005).

Chromosome replication in UV-irradiated cells

To investigate the effect of UV on DNA replication and chromosome segregation we used a strain in which ori-

gin and terminus areas of the chromosome were tagged with *lacO* and *tetO* arrays, respectively (Fig. 1B), carrying a plasmid encoding LacI-eCFP (enhanced cyan fluorescent protein) and TetR-eYFP (enhanced yellow fluorescent protein) repressors to decorate these arrays (Lau et al. 2003). We first addressed the question of replication fork progression. If a fork meets a pyrimidine dimer, fast reinitiation downstream from the lesion, as predicted by Rupp and Howard-Flanders (1968), should enable the cell to replicate the array near the terminus region. However, if replication is stalled and the replication fork has to be processed in a time-consuming way, cells should remain in a state in which the origin array may have been duplicated, but the terminus array remains as a single focus.

Cells were irradiated with 30 J/m² UV, which has been estimated to induce ~1200 lesions per chromosome, which translates to one lesion every 8 kb per single strand (Appendix I in Materials and Methods; Sedgwick 1975; Courcelle et al. 2006). At least 80% of the cells survived the exposure. Without irradiation, cells had morphologies typical of exponential growth in broth: 13.2% showed two foci for the origin region, 33.1% had three, 50.4% had four, and 3.3% had five. Most (87.6%) showed one focus for the terminus region, the remainder had two (Fig. 2A; Lau et al. 2003). The overall ratio of origin to terminus foci was 3:1. By 30 min after irradiation, all cells were filamentous and there was little sign of any increase in terminus foci. The number of discrete origin foci per cell was also largely unchanged but these showed an increase in intensity (Supplementary Fig. S1). This effect became even more striking by 60 min, but the numbers of terminus foci were still quite low: 82.2% had one to four origin foci, with an average of 2.9, but the majority (98%) had only one to two terminus foci, with an average of 1.6 (Fig. 2A). The increase in intensity of the origin foci was a feature of 81% of the filaments at this stage. By 90 min, the high intensity of the origin foci had largely disappeared, but the number of discrete origin foci per filament had increased substantially. The mean overall was 12.5, with 75% showing seven to 17 and 9.7% showing 19–23. Furthermore, these foci were spreading out along the filaments (Fig. 2A,B). This number is in line with the origin firing occurring roughly every 30 min, which corresponds to a measured doubling time of 30.4 min for the nonirradiated cells. These data demonstrate that UV irradiation does not prevent origin firing, which is consistent with previous studies (Billen 1969). More importantly, they show that the origin continues to fire at the normal rate in the majority of cells, which excludes the existence of a eukaryote-like G₁-S transition checkpoint. There is no evidence that UV induces DnaA-dependent *oriC* firing.

In contrast, the number of terminus foci per filament at 90 min remained low, with an overall average of 2.4 (Fig. 2A,B). By 120–150 min, the pattern had changed dramatically. The number of terminus foci increased to an average of 4.6 per filament overall and in 45.3% of cases ranged from five to 13. Furthermore, these had interspersed with the origin foci (Fig. 2B, right panel). From

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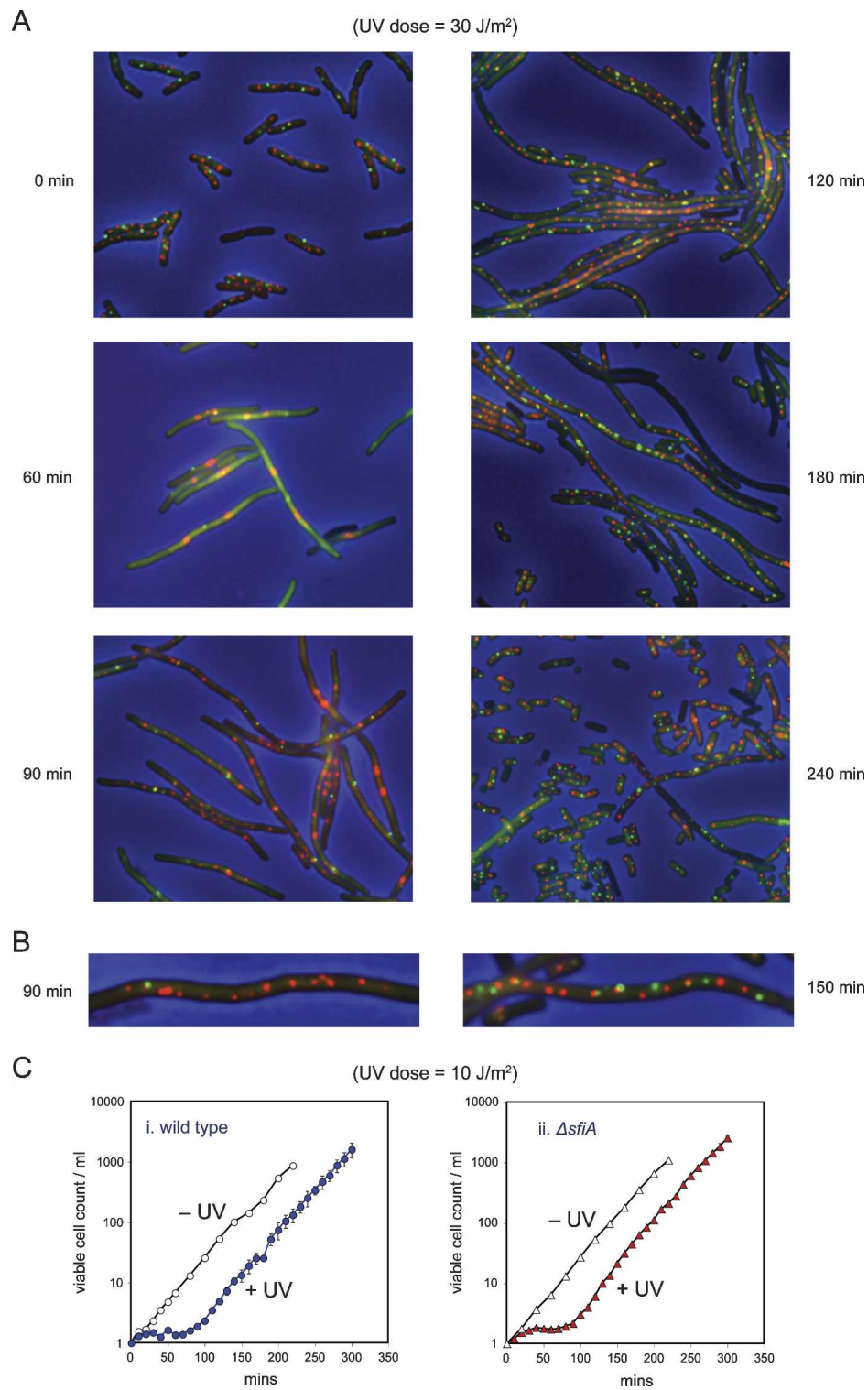


Figure 2. Effect of UV on cell cycle progression. (A) Fluorescence microscopy showing replication of origin (red foci) and terminus (green foci) areas of the chromosome (combined phase contrast and fluorescence images are shown). The strain used was APS345. The incubation time after irradiation is indicated. (B) Enlargements of filaments from a repeat of the experiment in A. (C) Viable cell replication following irradiation. The strains used were MG1655 (wild type) and N5209 (*sfiA11*). Data for the irradiated cells are the mean (\pm SE) of three experiments. The data for the nonirradiated cells are the mean of two experiments that gave almost identical values.

180 min onward, more and more normal-sized cells began to appear, and by 240 min there were few filaments remaining. By this time cells became quite short, indicating they were entering stationary phase. The ratio of origin to terminus foci was reduced to 1.6:1. We repeated the experiment using a UV dose of 10 J/m^2 , which increases the interlesion distance per strand to ~24 kb. A very similar response was seen, except that the increase in the intensity of the origin foci was less dramatic, and recovery occurred earlier (Supplementary Fig. S1).

One explanation for these data would be that ongoing DNA replication is blocked for a period in UV-irradiated cells, preventing any increase in the number of terminus foci. During this delay, *oriC* continues firing at about the rate in nonirradiated cells, with the result that foci for the region near the origin accumulate in situ. However, the blocked forks do ultimately recover, or are rescued, enabling the newly replicated DNA molecules, and hence the origin foci, to separate. Finally, the chromosome is duplicated and the cells divide.

The cells used might filament and delay septation abnormally because of the arrays and plasmid. (Note: Repressors were induced only in the samples of irradiated cells taken for analysis.) We therefore examined the time taken for irradiated cells lacking these elements to resume division after UV. Division was delayed by 60–70 min after a dose of 10 J/m^2 (Fig. 2C, panel i). This delay was also observed by time-lapse microscopy (data not shown). An almost identical delay was observed in a *sfiA* mutant lacking the SOS-induced division inhibitor (Fig. 2C, panel ii; data not shown). These observations indicate that SOS-induced cell filamentation is not the major reason why division takes so long to resume. They support the idea that replication of the terminus, and hence chromosome segregation, is much delayed.

To investigate whether the increase in the origin signal is indeed due to replication, we repeated the experiment with a *dnaC7* temperature-sensitive derivative. DnaC binds DnaB and is necessary for loading DnaB both during replication initiation at *oriC* and during rescue of stalled forks by PriA/PriC (Marians 2004). DnaC was inactivated by shifting the cells to 42°C directly after irradiation with 10 J/m^2 . The cells filamented, but in sharp contrast to what was seen with *dnaC*⁺ cells there was almost no change in the number of origin and terminus foci (Supplementary Fig. S2). This confirms that replication is responsible for the increase in the number of foci for the origin region.

Replication after UV irradiation requires loading of the DnaB replicative helicase

To gain a more quantitative measure of how UV affects new initiation at *oriC*, we measured incorporation of [³H]thymidine in both wild-type control and temperature-sensitive *dnaA* and *dnaC* strains shifted to 42°C immediately after irradiation. In the control, the rate of incorporation after UV was reduced to an extent consis-

tent with synthesis being delayed for some 10–15 min (Fig. 3A; Khidhir et al. 1985; Courcelle et al. 2003). In mock-irradiated *dnaC7* cells, incorporation continued for a time before reaching a plateau consistent with synthesis by all existing replication forks coming to an end. However, hardly any incorporation was detected after UV (Fig. 3A). Thus, even though UV lesions are not expected to block advance of DnaB, it would appear that little or no synthesis is possible without the means to load the replicative helicase. This implies that existing replisomes are unable to continue past any significant number of pyrimidine dimers. The fact that we did not detect residual synthesis associated with fork progression to the first blocking lesion likely reflects the delay between irradiation and addition of label. It reinforces the idea that the existing forks proceed past very few lesions, and indeed many may halt at the first lesion encountered. However, this result alone does not exclude the possibility that replication continues with only slight hindrance, as suggested by Heller and Marians (2006), since assembly of new replisomes downstream from lesions may be rapid.

UV irradiation induces DnaA-independent DNA synthesis

As with *dnaC*, nonirradiated *dnaA46* cells incorporated thymidine to an extent consistent with completion of existing rounds of replication (Fig. 3B). However, incorporation was significantly greater in the irradiated cells, as also reported by Jonczyk and Ciesla (1979), contrasting sharply with the *dnaC7* result (Fig. 3A). Essentially identical results were obtained using strains carrying temperature-sensitive *dnaA167* or *dnaA204* alleles (Fig. 3C). Since all synthesis after UV irradiation depends on DnaC, we assume the synthesis induced by UV and detected in irradiated *dnaA* temperature-sensitive cells requires DnaB loading. We suspect it reflects the establishment of new replication forks via the initiation of stable DNA replication, which is known to be DnaA-independent and triggered by DNA damage (Kogoma 1997). This would be consistent with the fact that, after 70-min incubation, the irradiated cells have incorporated more than twice the amount of [³H]thymidine into acid-precipitable material than the nonirradiated cells. Without new forks, it is difficult to see how this extra synthesis could be achieved. Excision repair is highly unlikely to be sufficient given the number of lesions introduced and the known lengths of the repair tracts. Indeed, we found that this UV-induced synthesis is detectable in an excision repair-defective mutant (data not shown). However, there is clearly less synthesis after UV in the *dnaA* strains than in the wild type (Fig. 3B,C). Taken together, these data indicate that DnaA-dependent *oriC* firing and UV-induced, DnaA-independent synthesis are responsible for a substantial fraction of the synthesis seen after irradiation of wild-type cells. They are also consistent with the evidence that the origin can fire when the terminus cannot replicate (Fig. 2).

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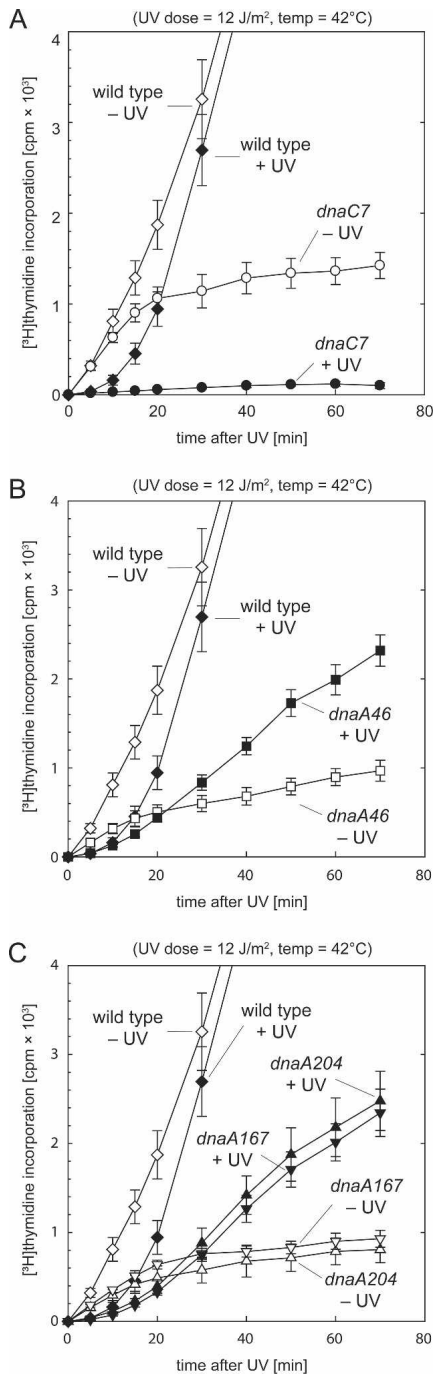


Figure 3. Effect of UV on DNA synthesis in *dnaA46* and *dnaC7* strains. (A) $[^3\text{H}]$ thymidine incorporation in wild-type (N1141) and *dnaC7* (AU1080) cells. Data are the mean (\pm SE) of three experiments. (B) $[^3\text{H}]$ thymidine incorporation in wild-type (N1141) and *dnaA46* (AU1068) cells. Data are the mean (\pm SE) of four to five experiments. The data for the wild type are reproduced from A for comparison. (C) $[^3\text{H}]$ thymidine incorporation in wild-type (N1141), *dnaA167* (AU1093), and *dnaA204* (AU1094) cells. Data are the mean (\pm SE) of three to four experiments. The data for the wild type are reproduced from A for comparison.

5-Bromo-2'-deoxyuridine (BrdU) labeling reveals a transient delay in progression of all pre-existing replication forks

The incorporation of $[^3\text{H}]$ thymidine provides no indication of whether there is disproportionate synthesis at any particular chromosomal location. To gain a more detailed picture of where synthesis is taking place, we labeled new DNA with BrdU, digested the chromosome with NotI, and separated the fragments by PFGE before probing for BrdU. In an exponential culture of nonirradiated cells, fragments should incorporate BrdU to a level reflecting fork distribution in the asynchronous cell population and the length of each fragment. This is what we observed; signal intensity in the fragments increased as predicted over a 15-min period (Fig. 4B).

A very different picture emerged when the cells were irradiated before adding BrdU. The signal detected during the first 15–20 min was much reduced for all fragments (Fig. 4B). This indicates a delay in the progression of all replication forks, as suggested by the reduced rate of thymidine incorporation (Fig. 3).

Furthermore, the bands containing DNA fragments located at or close to *oriC* appeared to give an even stronger signal than expected at early times when compared with those containing only *oriC*-distal fragments (Fig. 4A,B, green arrows). This is most easily observed with the band labeled I. This band reflects BrdU incorporation into three NotI fragments—two located near *oriC* and one ~1.9 Mbp away; i.e., ~85% of the distance to *ter* (Fig. 4A). Because these are relatively small (~30–40 kb) and well separated from other fragments, they could be resolved using different gel running conditions. Resolution of these fragments showed an early and increased level of BrdU labeling of the two closest to *oriC*. Labeling of the third fragment beginning 1.89 Mbp away from *oriC* is much delayed (Fig. 4D). We quantified the BrdU in these fragments and calculated the ratio of the label per kilobase in the origin-proximal and origin-distal regions. Without UV, the ratio averaged 1.7:1. With UV, it increased gradually to a maximum of 16:1 at 35 min, before reducing to 4.9:1 at 40 min. This quantification confirms that UV delays progression of pre-existing forks but does not prevent *oriC* from firing. Because of their large size (≥ 200 kb), and a difference of only 2–7 kb between them, the individual fragments contributing to the two intermediate bands labeled in Figure 4A cannot be resolved by PFGE to allow similar quantification of the BrdU incorporated into origin-proximal and origin-distal regions. The one unique DNA fragment (the slowest-migrating band indicated by arrow in Fig. 4A) covers so much of the chromosome that it is uninformative.

To determine whether the increased labeling of the most origin-proximal fragments is due to DnaA-dependent *oriC* firing, we conducted the same experiment with a *dnaA46* strain, shifting the cells to 42°C directly after irradiation. All fragments showed BrdU incorporation after an initial delay, similar to that seen with wild-type cells (Fig. 4C). This confirms our $[^3\text{H}]$ thymidine incorporation data showing that replication is not com-

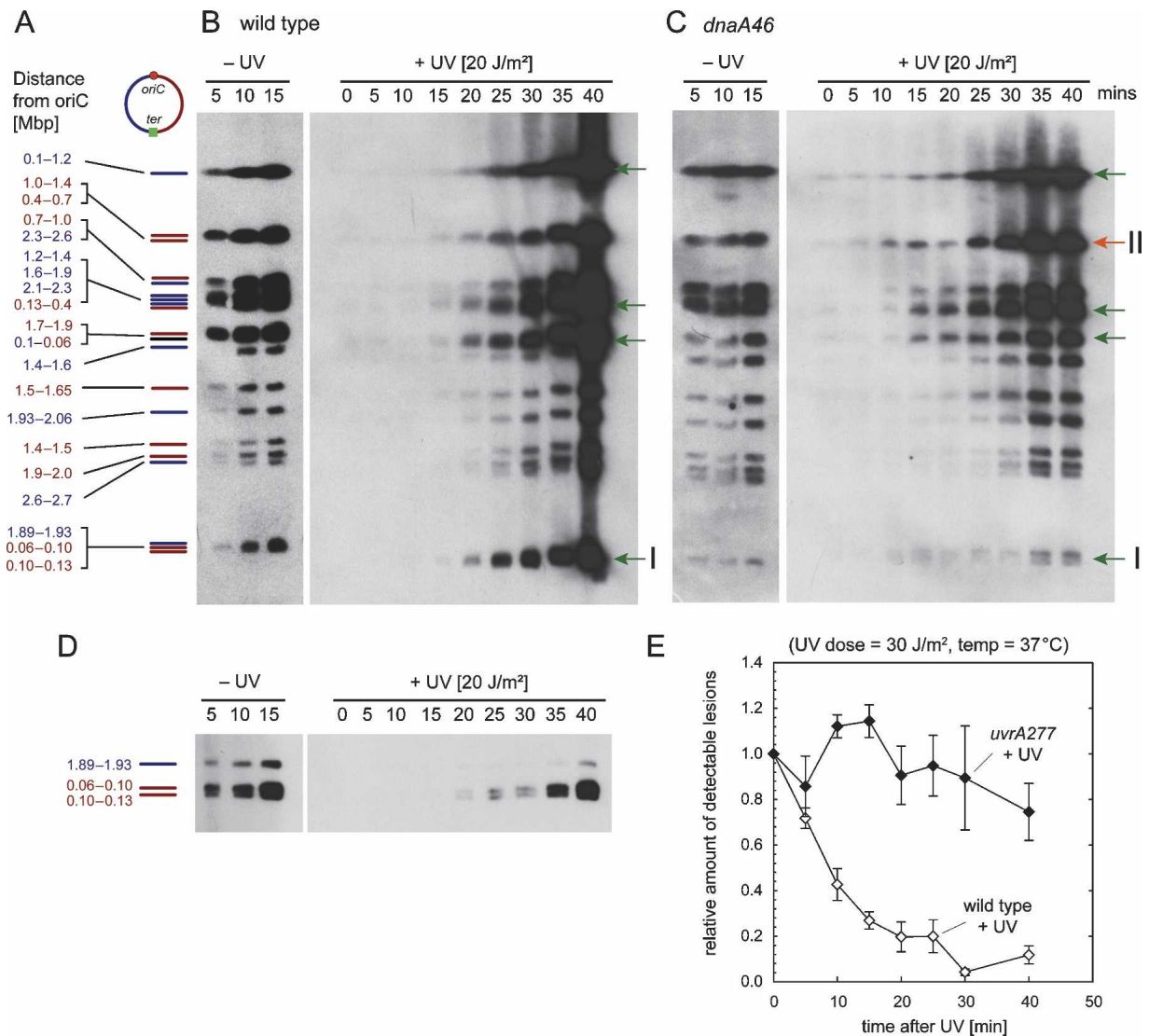


Figure 4. Replication and repair of UV-irradiated DNA. (A) Schematic NotI restriction pattern of the *E. coli* chromosome. The distance from *oriC* to each end of the fragments is indicated. Fragments clockwise and counterclockwise of *oriC* are shown in red and blue, respectively. (B) Fluorograph showing BrdU incorporation into the chromosome of wild-type strain MG1655 ±UV. Origin-proximal bands labeled intensively are identified with green arrows. (C) BrdU incorporation pattern in *dnaA46* strain AU1054. To inhibit *oriC* firing, cells were shifted to 42°C directly before adding BrdU. (D) Resolution of band I identified in B. (E) Pyrimidine dimer removal from strains MG1655 (wild type) and N4280 (*uvrA*).

pletely blocked. However, the disproportionate labeling of the *oriC*-proximal fragments, especially those contributing to band I, was no longer evident. The weak labeling of fragments contributing to band I in the nonirradiated cells is consistent with the absence of origin firing at 42°C. These results indicate that after irradiation new rounds of replication are initiated from *oriC* in a normal DnaA-dependent manner. We could find no conclusive evidence that the UV-induced and DnaA-independent synthesis revealed by [³H]thymidine incorporation (Fig. 3B) is initiated at sites like the *oriMs* within *oriC* described by Kogoma (1997). However, we cannot rigorously exclude the possibility as there is some indication of disproportionate labeling at early times of fragments

within three of the slower-migrating bands identified with green arrows in Figure 4C, one of which spans the *oriC* region. We were unable to sufficiently resolve these bands for a more quantitative analysis. If the *oriMs* do fire, the lack of early labeling of band I would suggest that the resulting synthesis does not extend far in the clockwise direction. The band identified with an orange arrow and labeled II in Figure 4C shows perhaps a clearer indication of disproportionate signal at early times. Two NotI fragments migrate in this position, both of which would be replicated by the fork moving clockwise from *oriC* (Fig. 4A). So, it is possible that UV may induce initiation of DNA replication in either or both of these fragments.

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Taken together with the results shown in Figures 2 and 3, these data indicate that DNA synthesis by pre-existing replisomes is brought to a halt following the introduction of UV lesions into the DNA. The origin of replication keeps on firing and, after a delay, replication can be detected at all sites around the chromosome. The precise location of the additional UV-induced, DnaA-independent synthesis (Fig. 3B) is not clear from the BrdU labeling. However, all the replication depends on DnaC, which indicates that it is carried out by new replisomes assembled via DnaB loading. Little or none of it is carried out by replisomes present at the time of irradiation.

DNA synthesis is reduced drastically in the absence of excision repair and origin firing

Can we estimate how long ongoing replication is delayed? Rupp and Howard-Flanders (1968) used [³H]thymidine accumulation into the DNA to conclude that replication forks are delayed by no more than ~10 sec per lesion (Heller and Marians 2006). However, we have shown there is continued origin firing in UV-irradiated cells and also a significant amount of UV-induced synthesis. The accumulation of [³H]thymidine into DNA is therefore likely to seriously underestimate the delay. But the Rupp and Howard-Flanders estimate was based on studies with excision repair-defective *uvrA* cells. Therefore, we compared the rate of accumulation of [³H]thymidine in *uvrA* and *dnaA46 uvrA* backgrounds shifted to 42°C after UV. Nonirradiated *uvrA* cells accumulated label at about the same rate as a wild-type strain (cf. Figs. 3A and 5A). After UV, accumulation was reduced drastically and, at the same dose (12 J/m²), to a level lower than in the *dnaA46* strain (Fig. 5A; Supplementary Fig. S3B). In the *dnaA uvrA* double mutant, the accumulation was even lower (Fig. 5A). This shows very clearly that the delay in progression of ongoing forks induced by UV cannot be calculated simply by looking at total [³H]thymidine incorporation (see also Appendix I in Materials and Methods).

The origin to terminus ratio increases after UV irradiation

The fluorescent images shown in Figure 2A demonstrate there is a substantial delay of ongoing replication. They indicate that the number of termini in UV-irradiated cells is still very low up to 90 min after the irradiation, but increases rapidly between 90 and 120 min. However, this observation might also be explained if replication forks proceeded through to the terminus region without hindrance but segregation of the replicated termini was delayed. To address this possibility, we investigated the ratio of two chromosomal loci by Southern analysis. Two probes were used, one binding within *mioC*, which contains *oriC*, and one in *ribA*, which lies next to *terA*. To facilitate the analysis, a *dnaC7* strain was used and synchronized by a shift to 42°C for 45 min prior to irradiation.

As expected, the nonirradiated control showed an increase in the origin signal relative to the terminus signal,

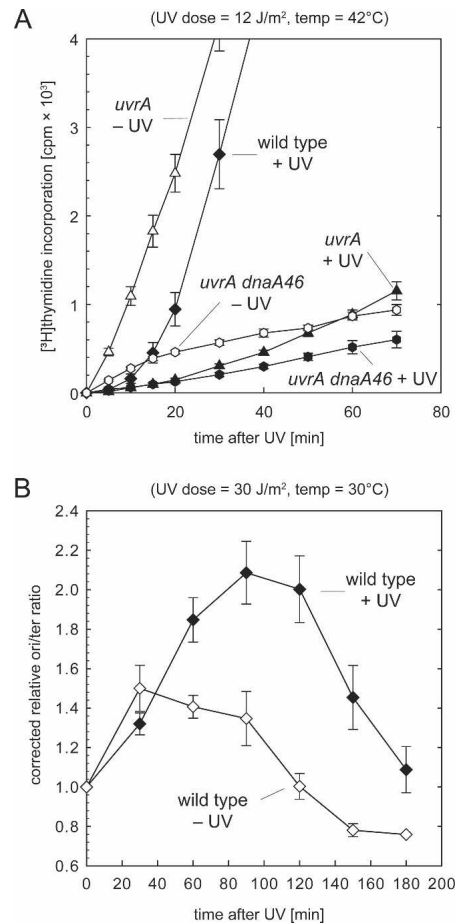


Figure 5. Effect of UV on DNA synthesis and chromosome replication. (A) [³H]thymidine incorporation in *uvrA* (AU1075) and *uvrA dnaA46* (AU1072) cells. Data are the mean (\pm SE) of three experiments. Data for irradiated wild-type (N1141) cells (Fig. 3A) are included for comparison. (B) Changes in the origin to terminus ratio during incubation of irradiated and nonirradiated cells. The strain was RCe79 (*dnaC7*). Cells grown at 30°C were synchronized by incubation at 42°C for 45 min before irradiation and shifting back to 30°C. Data are the mean (\pm SE) of three experiments.

reaching a ratio of 1.5 (Fig. 5B). The theoretical ratio should be ~2 for cells growing at 30°C, but microscopic analysis revealed that the synchrony of the starting material was not complete (data not shown). The very first value, set to one, is therefore an underrepresentation of the real ratio. The ratio stayed roughly at 1.5 over 90 min and decreased after 3 h to ~0.7, which represents the cessation of replication as cells enter stationary phase. The value of <1 reflects the initial underestimation.

The origin/terminus ratio also increased after UV, but with a slight initial delay (Fig. 5B). However, unlike in the nonirradiated cells it continued to increase, indicating that the number of origins increases faster than the number of termini. After 120 min, the ratio decreased. This pattern is in excellent agreement with the fluorescence microscopy data, showing that multiple termini foci appear between 90 and 120 min after irradiation.

Therefore, it seems reasonable to conclude that ongoing replication is blocked and hence the terminus cannot be duplicated.

The BrdU incorporation experiments (Fig. 4) provide some additional insight into the extent of the delay. After a UV dose of 20 J/m² almost all of the bands that do not contain DNA fragments near *oriC* show at most a marginal increase in signal intensity for at least 15–20 min (Fig. 4B). Surprisingly, there seems to be quite rapid BrdU incorporation into all the fragments after this period. This does not require DnaA since the time course of BrdU incorporation looks very similar in a *dnaA46* mutant at 42°C (Fig. 4C). However, synthesis depends almost completely on DnaC (Fig. 3A; Supplementary Fig. S3A). So we can conclude there is a delay of at least 15–20 min before ongoing synthesis resumes.

Replication resumes at a time when most UV-induced lesions have been removed

What could be the reason for the apparent synchrony of replication restart revealed by BrdU incorporation, and the subsequent continuation of synthesis at a rate comparable to that in nonirradiated cells? One obvious process would be fork rescue coupled with removal of the UV-induced lesions. We therefore determined the rate of thymidine dimer removal in wild-type cells after a 30-J/m² dose. This revealed that ~80% of dimers are removed within 20 min (Fig. 4E), which is in good agreement with published data (Courcelle et al. 1999). Thus restart of stalled forks and damage removal may be closely coupled.

The data we have presented so far indicate that [³H]thymidine incorporation may be misleading in terms of the rate of ongoing replication because new initiation events at *oriC* and UV-induced synthesis contribute substantially to the total incorporation observed. Variation in the extent of origin firing may explain the reported differences in the amounts of DNA synthesis detected in UV-irradiated *uvrA* cells (Rupp and Howard-Flanders 1968; Courcelle et al. 2005, 2006).

If DNA synthesis in UV-irradiated *uvrA* cells were to have difficulty recovering, as suggested (Courcelle et al. 2005, 2006), only those cells lacking any lesions between the fork and the terminus should complete replication. These should represent a minority of the total after UV doses introducing a substantial number of lesions. To address this possibility, we examined the origin to terminus ratio. Figure 6A shows that after a dose of 5 J/m² an *uvrA* strain carrying origin and terminus arrays shows intense origin foci, as seen with the wild type at higher doses (Fig. 2A; Supplementary Fig. S1). However, the cells continued to filament throughout the 240-min post-irradiation incubation and there is no evidence of the regular dispersal of origin foci along these filaments or of their subsequent interspersions with replicated terminus foci (cf. Figs. 6A and 2B). Some filaments do ultimately show several foci for the terminus region, indicating that some replication of the terminus is possible, but this replication occurs much later than in wild-type cells (Fig. 6A; Supplementary Fig. S1).

The increase in the origin to terminus ratio was confirmed by Southern analysis. The ratio increased to ~3.5 by 120 min and then remained constant (Fig. 6B). That it does not reduce as in the wild type reinforces the idea that most *uvrA* cells have great difficulty replicating the terminus. This would be consistent with the fact that only 1.5% survive a UV dose of 5 J/m² (data not shown).

However, some multiplication of the terminus area is evident. To investigate whether this is due to generalized progression of replication forks or to some UV-induced replication initiated specifically near the terminus (Kogoma 1997), we examined the pattern of BrdU incorporation. Figure 6C shows substantial incorporation close to *oriC*, confirming that the origin continues to fire. There is also incorporation into more distally located fragments, including those at or near the terminus. The PFGE resolved several such fragments (identified with a bracket in Fig. 6C). However, their labeling is much delayed—much more so than is seen in wild-type cells despite the fourfold lower UV dose (note the different time scales in Figs. 4B, 6C). Thus, even without excision repair, some replication is able to resume to complete chromosome duplication, though this is much delayed. This fits with the observation that 37% of *uvrA* cells survive a UV dose introducing ~50 dimers per chromosome (Rupp and Howard-Flanders 1968), or only 30–40 dimers per chromosome according to the number of lesions generated per joule estimated by Sedgwick (1975) and Courcelle et al. (2006).

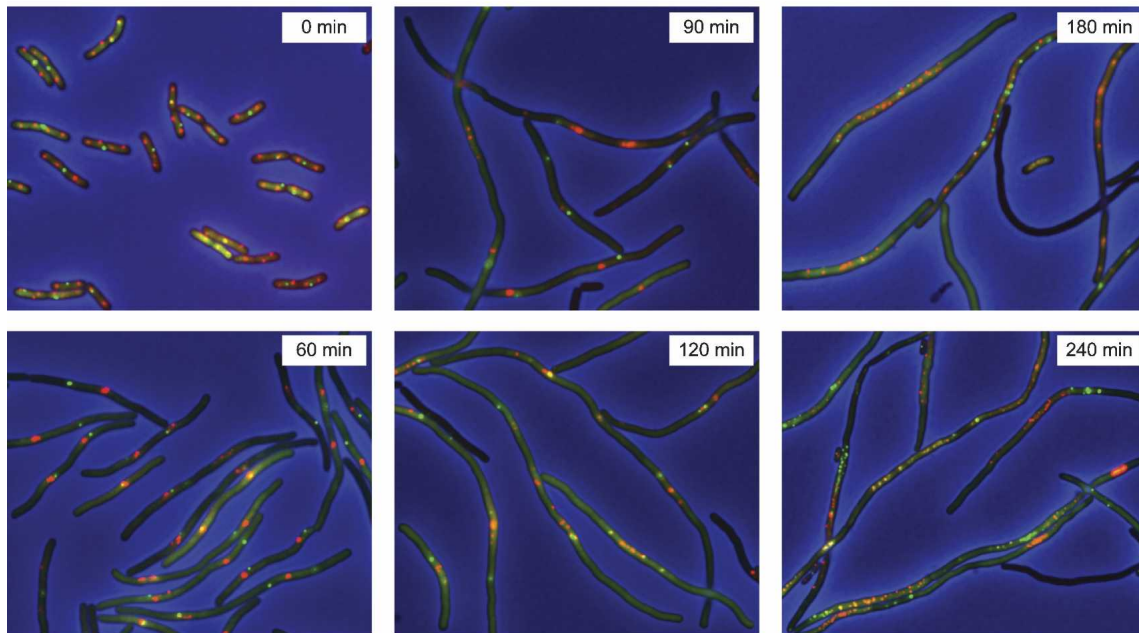
What happens in *uvrA* cells if the lesion density is high? Figure 6B shows that the origin to terminus ratio increased far more slowly when the UV dose was increased from 5 J/m² to 30 J/m², and never decreased, indicating that there is much less origin firing or that the duplicated DNA is degraded. We assume this reflects the higher incidence of damage at or close to *oriC*, which does not allow forks coming from the origin to progress very far and thus limits the ability of the origin to fire. A high lesion density in combination with a lack of repair therefore might directly influence the capability of origin firing. Consistent with this, the *uvrA* array strain showed a low number of both origin and terminus foci even after 180 min post-UV (Supplementary Fig. S2). After prolonged incubation the filaments seem to accumulate dispersed aggregations of the fluorescent repressors rather than foci.

Taken together, the data we have presented indicate that the substantial DNA synthesis associated with origin firing seen in wild-type cells depends on nucleotide excision repair. This is consistent with the results described by Courcelle et al. (2005, 2006). Our results emphasize the importance of eliminating origin firing and UV-induced synthesis when evaluating the progression of pre-existing replication forks in UV-irradiated cells.

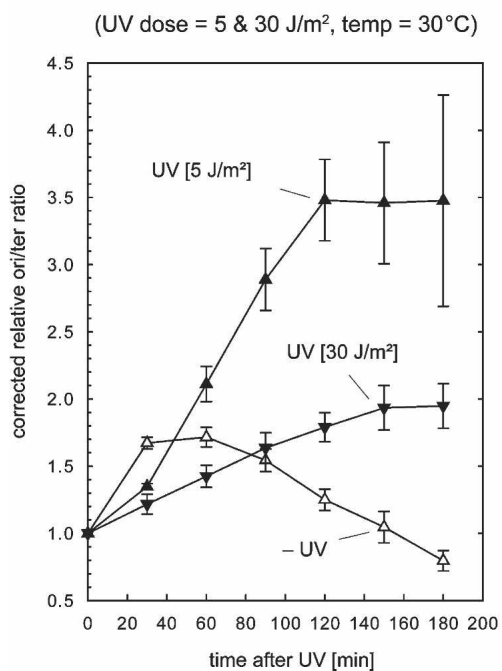
Discussion

We revisited the question of what happens to replication forks when they encounter UV lesions in the template DNA. The data presented demonstrate that replication

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A (UV dose = 5 J/m²)

B



C

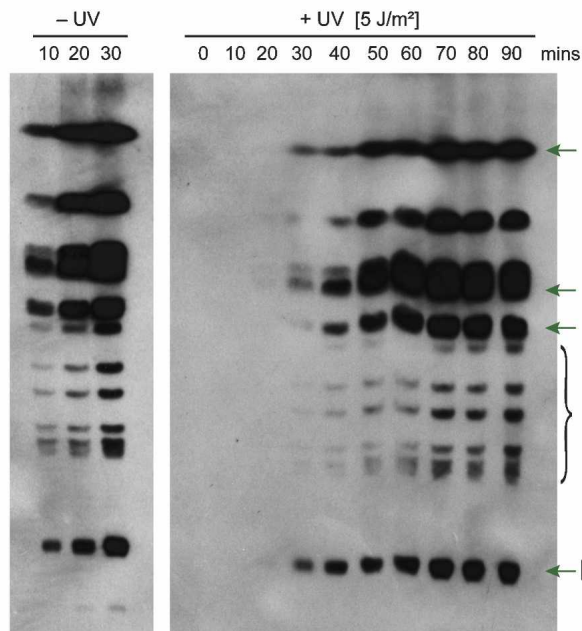


Figure 6. Effect of UV lesions on cell cycle progression and DNA synthesis in the absence of DNA excision repair. (A) Fluorescence microscopy showing replication of origin (red foci) and terminus (green foci) areas of the chromosome. The strain was RCE129 (*uvrA*). (B) Changes in the origin to terminus ratio during incubation of irradiated and nonirradiated cells. The strain was RCE120 (*dnaC7 uvrA*). Cells grown at 30°C were synchronized by incubation at 42°C for 45 min before irradiation and shifting back to 30°C. Data are the mean (\pm SE) of three or more experiments. (C) Fluorograph showing the time course and pattern of BrdU incorporation into the chromosome of *uvrA* strain N4280 with or without UV irradiation as indicated.

stops for a minimum of 15–20 min, or at least slows down dramatically, before resuming at the original rate. The data also show that undamaged origins continue to

fire and that UV also induces synthesis that is independent of the initiator protein, DnaA. Our observation that replication restart occurs when most lesions have been

removed raises the possibility that replication restart and lesion removal are coupled. Replication and cell division suffer tremendous delays if lesion removal is prevented. Finally, we demonstrate that essentially all the synthesis seen after irradiation depends on DnaC, which is required to load the DnaB replicative helicase.

These data fit well with models invoking the stalling of forks at UV lesions and their extensive processing before replication can restart (Fig. 1A, ii). These models have been reviewed recently (McGlynn and Lloyd 2002). Essentially, these models propose that a replication fork may skip over a lesion in the lagging strand template, leaving a gap to be filled by recombination. But when it encounters a polymerase-blocking lesion in the leading strand template, the two polymerases are decoupled. Fork progression coupled with continued extension of the lagging strand exposes the leading strand template (Higuchi et al. 2003; Pages and Fuchs 2003; McInerney and O'Donnell 2004). RecA loads on this exposed strand, forming a nucleoprotein filament that acts both to induce the SOS repair response and to provide means to process and rescue the damaged fork. After a delay associated with restoration of the fork, replication resumes when either PriA or PriC loads DnaB to enable assembly of a new replisome (Fig. 1A, ii).

In the meantime, SOS induction will have led to an early and rapid increase in the proteins (UvrA and UvrB) needed to initiate nucleotide excision repair, enabling most of the lesions in the chromosome to be removed rapidly. Therefore, by the time replication is able to restart, it will be able to continue with a minimum of further impediment. Our results demonstrate that replication resumes at all sites in the chromosome at a rate commensurate with that in nonirradiated cells (Fig. 4B), consistent with this model.

Without means to remove lesions, *uvrA* cells would face difficulties consistent with their extreme UV sensitivity. The much-delayed DNA synthesis in these cells (Figs. 5A, 6; Rupp and Howard-Flanders 1968; Courcelle et al. 2005, 2006) is consistent with replication forks stuttering at lesion after lesion, and with a need to reassemble a replisome each time. The cells that survive at low doses presumably do so through a combination of template switching, gap filling by recombination, and possibly translesion synthesis, aided perhaps by the SOS-induced elevation of the associated activities.

This view of events in UV-irradiated cells stands in sharp contrast to the idea that forks proceed largely unhindered, skipping over lesion after lesion and leaving many gaps in their wake to be filled by recombination (Fig. 1A, i), as illustrated originally by Rupp and Howard-Flanders (1968). Our studies revealed that [³H]thymidine is incorporated into the DNA of UV-irradiated *uvrA* cells with a delay close to the estimate made by Rupp and Howard-Flanders (1968) (see Appendix I in Materials and Methods). However, the evidence of origin firing and UV-induced synthesis revealed by our studies demonstrates clearly that averaging the delay over the number of lesions is very misleading. Pages and Fuchs (2003) observed a delay in replicating past a single leading strand

block corresponding to about one cell cycle. Our estimates of the delay caused by UV are in accord with this observation.

Many inferences have been drawn from the inverse relationship between the length of newly synthesized DNA and the UV dose, and the fact that the molecular weight of the new DNA strands increased during post-irradiation incubation (Rupp and Howard-Flanders 1968; Bridges and Sedgwick 1974). However, new strands extended by refiring of the origin or by UV-induced initiation at other sites would have these same two properties. Our studies suggest such strands might comprise a substantial fraction of the newly synthesized DNA.

Our results do not eliminate the idea that a fork may skip lesions, even some on the leading strand (Heller and Marians 2006), leaving gaps to be filled by recombination. Iyer and Rupp (1971) demonstrated there are gaps in DNA made during growth of a UV-irradiated *uvrA* strain but did not determine whether these were present in both nascent strands. At least one gap is likely when the first lesion encountered is in the lagging strand template. However, the delay resulting from subsequent fork stalling at a lesion in the leading strand, coupled with the rapid removal of lesions during this period, makes it likely wild-type cells would have to deal with few gaps on the whole. Our assays are not sufficiently sensitive to estimate how many on average.

The observed delay before replication can resume suggests extensive processing of stalled forks, but why this should take at least 15 min, and possibly much longer, is not clear. A possible clue comes from the need for DnaB loading. It may simply take time to assemble new replisomes. Dissociation of stalled replisomes may inactivate one or more key components, or these components may be in limited supply as a part of normal cell cycle regulation. Increased demand for these proteins may also result from the continuing *oriC* firing and UV-induced initiations. Furthermore, the processing of stalled forks might be slow. It has been suggested that RecA might be involved in the stabilization and/or reversal of stalled replication forks (Courcelle and Hanawalt 2003), and it has been shown that the strand exchange reaction promoted by RecA *in vitro* is relatively slow (Camerini-Otero and Hsieh 1993; Voloshin and Camerini-Otero 2004). Stabilization or processing of stalled forks by RecA could therefore take considerable time. Even if replisomes are reassembled or processed quickly, it is possible cells deliberately slow down replication restart (Opperman et al. 1999; McInerney and O'Donnell 2004).

Delaying replication restart while allowing origin firing may be of selective advantage in a rapidly dividing cell population exposed to DNA damage. It would facilitate safe removal of any lesions and enable a cluster of replication forks to create multiple copies of the genome once the lesions are removed, and subsequently the equivalent number of viable cells. This would compensate somewhat for any delay caused initially by the blocking lesion.

By providing time to clear the path for replication to resume, the delay in replication restart also eliminates a

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second objection to the Rupp and Howard-Flanders model. Wild-type *E. coli* cells withstand UV doses that introduce a thousand or more pyrimidine dimers into the chromosome with little reduction in survival and only a modest increase in mutation. If replication forks skip past many of these lesions, as in the Rupp and Howard-Flanders model, an inordinate number of recombination events might be required to close all the gaps left behind, even if some lesions were removed by excision repair. High levels of recombination are known to be destabilizing for the genome because they can elicit illegitimate exchanges and also because the intermediates delay chromosome segregation and cell septation. This is evident from the high mutation rates and general debility of “hyper-rec” mutants, such as those lacking UvrD helicase (Arthur and Lloyd 1980; Lloyd 1983; Bierne et al. 1997). Mutations that elevate mitotic recombination in eukaryotes have a similar destabilizing effect and those in humans are noted for their association with a much-elevated risk of cancer (Myung et al. 2001). Most eukaryotes appear to curb the activity of the Rad51 family of recombinases (Krejci et al. 2003; Veaute et al. 2003), except when efficient recombination is needed, as in meio-

sis (Nicolas et al. 1989). *E. coli* has also evolved mechanisms to curb RecA during normal growth (Flores et al. 2005; Mahdi et al. 2006). Thus, the delay in restarting replication revealed in our work may be yet another reflection of how advantageous it is to avoid recombination whenever possible.

Materials and methods

Bacterial strains

The studies described used derivatives of wild-type *E. coli* K-12 MG1655 (Table 1), with the exception of the [³H]thymidine incorporation assays when we employed strain N1141 and its derivatives. N1141 carries *thyA54* along with a *deo* mutation, allowing growth with low levels of thymine. For fluorescence microscopy, MG1655 derivatives carrying *lacO240* and *tetO240* arrays were transformed with pLAU53, which encodes arabinose-inducible LacI-eCFP and TetR-eYFP (Lau et al. 2003).

Media and general methods

LB broth and 56/2 salts media, and methods for monitoring cell growth and P1vir transduction and determining sensitivity to UV have been cited (McGlynn and Lloyd 2000).

Table 1. *E. coli* K-12 strains

Strain	Relevant genotype ^a	Source ^a
BW6164	<i>thr-43::Tn10</i>	CGSC
NY171	<i>deo-41 dnaC7</i>	CGSC
RUC663	<i>tnaA::Tn10 dnaA46</i>	Tove Atlung
SS1791	<i>tnaA300::Tn10 dnaA167</i>	Steve Sandler
SS2241	<i>tnaA300::Tn10 dnaA204</i>	Steve Sandler
AB1157 derivatives		
IL01	<i>attTn7::lacO240::kan</i>	David J. Sherratt
IL04	<i>zdd/e::tetO240::gen attTn7::lacO240::kan</i>	David J. Sherratt
N1141 derivatives		
N1141	F ⁻ <i>lacI3 lacZ118 metE70 leuB6 proC32 thyA54 deo(BC) malA38 ara-14 mtl-1 xyl-5 str-109 spc-15</i>	Low thymine requiring derivative of KB Low strain KL266
AU1068	<i>tnaA::Tn10 dnaA46(ts)</i>	P1.RUC663 × N1141 to Tc ^r
AU1072	<i>tnaA::Tn10 dnaA46 ΔuvrA::apra</i>	P1.N6024 × AU1068 to Apra ^r
AU1073	<i>thr-43::Tn10 dnaC7</i>	P1.N6594 × N1141 to Tc ^r
AU1075	<i>ΔuvrA::apra</i>	P1.N6024 × N1141 to Apra ^r
AU1080	<i>dnaC7 deo(BC)</i>	P1.N1141 × AU1073 to Thr ^r
AU1093	<i>tnaA::Tn10 dnaA167</i>	P1.SS1791 × N1141 to Tc ^r
AU1094	<i>tnaA::Tn10 dnaA204</i>	P1.SS2241 × N1141 to Tc ^r
MG1655 derivatives		
MG1655	F ⁻ <i>dnaC⁺ dnaA⁺ thr⁺ tnaA⁺ uvrA⁺</i>	Mahdi et al. 2006
APS301	<i>attTn7::lacO240::kan</i>	P1.IL01 × MG1655 to Km ^r
APS345	<i>attTn7::lacO240::kan zdd/e::tetO240::gen</i>	P1.IL04 × APS301 to Gen ^r
AU1054	<i>tnaA::Tn10 dnaA46</i>	P1.RUC663 × MG1655 to Tc ^r
N4280	<i>uvrA277::Tn10</i>	R.G. Lloyd, unpubl.
N6024	<i>ΔuvrA::apra</i>	R.G. Lloyd, unpubl.
N6594	<i>dnaC7 thr-43::Tn10</i>	P1.BW6164 × RCe79 to Tc ^r
RCe77	<i>thr-43::Tn10 attTn7::lacO240::kan zdd/e::tetO240::gen</i>	P1.RCe98 × APS345 to Tc ^r
RCe79	<i>dnaC7</i>	P1.NY171 × RCe98 to Thr ^r
RCe93	<i>dnaC7 attTn7::lacO240::kan zdd/e::tetO240::gen</i>	P1.RCe79 × RCe77 to Thr ^r
RCe98	<i>thr-43::Tn10</i>	P1.BW6164 × MG1655 to Tc ^r
RCe120	<i>dnaC7 uvrA277::Tn10</i>	P1.N4280 × RCe79 to Tc ^r
RCe129	<i>uvrA277::Tn10 attTn7::lacO240::kan zdd/e::tetO240::gen</i>	P1.N4280 × APS345 to Tc ^r

^aThe abbreviations *kan*, *apra*, and *gen* refer to insertions conferring resistance to kanamycin (Km^r), apramycin (Apra^r), and gentamycin (Gen^r), respectively. Tn10 confers resistance to tetracycline (Tc^r). Strains carrying *dnaA46*, *dnaA167*, *dnaA204*, or *dnaC7* are temperature sensitive for growth. (CGSC) Coli Genetic Stock Center, Yale University.

Fluorescence microscopy

Cells were grown to an A_{650} of 0.2 in LB broth supplemented with 0.5 mM IPTG and 40 ng/mL anhydrotetracycline to reduce repressor binding, without compromising focus formation. A 1-mL sample was removed and expression was induced at high levels by adding arabinose to 0.2%. The rest of the cells were pelleted, UV-irradiated on the surface of LB agar, and resuspended in the original, but filter-sterilized, supernatant to continue incubation before inducing further 1-mL samples at intervals thereafter. Arabinose-induced cells were transferred to a thin 1% LB agarose layer on microscopic slides, visualized with a BX-52 Olympus microscope equipped with a coolSNAP™HQ camera (Photometrics). eCFP and eYFP foci were visualized using the JP4-CFP-YFP filterset 86002v2 (Chroma). Images were taken and analyzed by MetaMorph 6.2 (Universal Imaging) and processed using MetaMorph and Adobe Photoshop.

Multiplication of cells surviving UV irradiation

To monitor recovery of cells surviving UV irradiation, cultures of the strains indicated were grown in LB broth and the cells were irradiated with UV as for fluorescence microscopy. The cells were resuspended in the original, but filter-sterilized, supernatant and diluted 10,000-fold in conditioned medium, which was created by growing the wild-type strain in fresh LB broth to an A_{650} of 0.2 with subsequent sterile filtration. The diluted cells were incubated in a 37°C shaking water bath and at each time point samples were removed, mixed with 2.5 mL of molten 0.6% top agar kept at 42°C, and plated on LB agar. At later time points, the samples were diluted a further 10- or 100-fold in conditioned medium before plating. Colonies were counted after incubation for 18–24 h at 37°C.

Measurement of DNA synthesis

Cultures were grown with vigorous shaking at 30°C in Davis medium [0.7% K_2HPO_4 , 0.3% KH_2PO_4 , 0.1% $(NH_4)_2SO_4$, 0.05% $Na_3C_6H_5O_7 \cdot H_2O$, 0.0001% thiamine, 0.4% glucose, 0.01% $MgSO_4$] supplemented with 1% casamino acids and 5 μ g/mL thymidine. At an A_{650} of 0.2, the cells were filtered onto 0.22 μ m cellulose acetate (Corning) and irradiated directly on the filter, or mock-irradiated, before resuspending in the filtrate. [3H]thymidine (specific activity 80.0 Ci/mmol, Amersham) was added to 2 μ Ci/mL before continuing incubation as indicated. Twenty-microliter samples were taken at intervals, applied to 2.5-cm² filters (Whatman 3MM), and immediately immersed in ice-cold 5% trichloroacetic acid for a minimum of 30 min. Filters were washed in three changes of fresh trichloroacetic acid and two of ethanol and dried, and the bound radioactivity was counted by liquid scintillation.

BrdU labeling

Cells were grown in 56/2 salts supplemented with 0.2% casamino acids and 0.32% glucose to an A_{650} of 0.2 and UV-irradiated as for fluorescence microscopy. Cells were resuspended in the sterile filtered supernatant and the first 2-mL sample was removed. BrdU (Sigma) was added to the rest of the culture to 20 μ g/mL. Two-milliliter samples were taken every 5 min, pelleted, and resuspended in 85 μ L TEE buffer (10 mM Tris-HCl, 10 mM EGTA, 100 mM EDTA at pH 8.0), containing 0.05% lauroylsarcosine. Eighty-five microliters of liquid 1% agarose in TEE buffer were added and the mixture was solidified in a disposable plug former (Bio-Rad) at 4°C. Plugs were treated with 5 mg/mL lysozyme in 3 mL of TEE buffer containing

0.05% lauroylsarcosine for 2 h at 37°C and then overnight at 52°C with 5 mg/mL proteinase K in 3 mL of TEE containing 1% SDS. Plugs were washed in TEE for 30 min at 37°C, treated with 1 mM phenylmethane sulphonyl fluoride in fresh TEE for 1 h at 37°C, and washed twice in fresh TEE for 30 min at 37°C. The plugs were subsequently transferred into 300 μ L of restriction enzyme buffer and incubated for 30 min at room temperature, the buffer was changed, and 25 U of NotI (New England Biolabs) was added. Chromosomal DNA was digested overnight and the fragments were separated on a 0.8% agarose gel in 0.5 \times TBE using a CHEF Mapper PFGE system (Bio-Rad) running with a gradient voltage of 6 V/cm, an included angle of 120°, and initial and final switch times of 1.65 and 32.45 sec, respectively, with a run time of 20 h at 14°C. For resolution of band I in Figure 4B the running conditions were the same, except the initial and final switch times were 1.65 and 2.43 sec, respectively. DNA was transferred to a Hybond-N⁺ Membrane (Amersham) by alkaline vacuum transfer and UV-cross-linked (120 mJ/cm²). After blocking with TBS (50 mM Tris-HCl, 150 mM NaCl at pH 8.0) containing 1% milk powder, the membrane was incubated for 2 h in the presence of mouse anti-BrdU antibody (Santa Cruz Biotechnology), diluted 1:5000 in TBS. Horse radish peroxidase-conjugated secondary antibody (goat anti-mouse, Bio-Rad) was used at a dilution of 1:10,000 for 1 h. The membrane was incubated with ECL Plus Western Blotting Detection Reagents (GE Healthcare) and the signal was visualized by exposure to X-Omat UV Plus film (Kodak).

Southern analysis of the origin to terminus ratio

Cultures of *dnaC7* strains were grown at 30°C in LB broth to an A_{650} of 0.2 and shifted for 45 min to 42°C for synchronization. Irradiation was as for fluorescence microscopy before continuing incubation at 30°C. Four-milliliter samples were removed at each time point. Cells were pelleted, resuspended in 500 μ L 10 mM Tris (pH 8.0), 10 mM NaCl, and 10 mM EDTA before adding 30 μ g RNaseA, 50 μ L Triton X-100 (10%), and 250 μ g of lysozyme, and incubated for 30 min at 37°C. Three-hundred micrograms of proteinase K were added and the sample was shifted to 65°C for 120 min. The DNA was extracted twice with phenol-chloroform and precipitated in 2.5 M ammonium acetate and 2 vol of ethanol. Purified DNA was resuspended in TE buffer. Three micrograms of chromosomal DNA of each sample were digested with XmaI and HpaI, and the fragments were resolved on a 0.7% agarose gel (45 V, 1 \times TAE, 16 h), transferred to a Zeta-Probe GT Membrane by alkaline vacuum transfer, and UV-cross-linked (120 mJ/cm²). One-hundred nanograms of each ^{32}P -labeled probe were annealed overnight at 65°C in 7 \times SSPE (150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA at pH 7.4), containing 60 mg/mL dextran sulphate and 1.2% SDS. Signal was visualized using a Kodak Storage Phosphor Screen, scanned with a STORM scanner system (Molecular Dynamics), and quantified with ImageQuant 5.2 (Molecular Dynamics). For calculation of the corrected relative origin to terminus ratio, the signal intensity of the origin signal was divided by the intensity of the terminus signal and all the ratios divided by the ratio of the very first sample directly after synchronization. Values are the mean of three experiments.

Thymine dimer removal

Cells were grown in LB broth and UV-irradiated as for fluorescence microscopy. Two-milliliter samples were removed, and DNA was extracted as for the Southern analyses and adjusted with TE to 250 μ g/mL. Following denaturation by boiling, 500- μ g samples were transferred to a Zeta-Probe GT Membrane via

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dot blot. DNA was baked on the membrane for 2 h at 80°C, probed with mouse anti-CPD antibody (Sigma), and diluted 1:1000, and complexes were detected with sheep anti-mouse, alkaline phosphatase-conjugated secondary antibody (Sigma), diluted 1:10,000 as described for detection of BrdU. Signal was developed using an AttoPhos AP Fluorescent Substrate System (Promega), measured using a STORM scanner system (Molecular Dynamics) in fluorescence mode (450-nm excitation, 520-nm emission), and quantified with ImageQuant 5.2.

Appendix I. Calculations of DNA synthesis delay in UV-irradiated cells and additional discussion

Calculations are based on the following assumptions:

- (1) Cells growing in minimal salts medium have two forks per cell on average.
- (2) Each fork takes ~40 min to traverse from *oriC* to *ter*.
- (3) Therefore, with a genome of ~4600 kb, replication proceeds in nonirradiated cells at a rate of ~2 kb/sec, or ~120 kb/min.
- (4) A dose of 1 J/m² UV introduced ~40 dimers per chromosome (Courcelle et al. 2006), which equates to one dimer every 115 kb. Therefore, a dose of 12 J/m² introduces a lesion every 9 kb or so, while a dose of 5 J/m² introduces a lesion about every 21 kb.

From the data in Figures 3A and 5A and Supplementary Figure S3B, which measured [³H]thymidine incorporation after a UV dose of 12 J/m² (or 5 J/m²) (Supplementary Fig. S3B), we estimated approximately how long it took for irradiated wild-type, *uvrA*, and *uvrA dnaA46* cells to incorporate the same amount of label as nonirradiated wild-type cells. The calculations were based on the value for *uvrA dnaA46* at 70 min and gave the following estimates for the delay in incorporation of the label in the UV-irradiated cells:

- (1) Nonirradiated wild type = 8 min.
- (2) Irradiated wild type = 16 min, a delay of 8 min.
- (3) Irradiated *uvrA* = 43 min, a delay of 35 min.
- (4) Irradiated *uvrA dnaA46* = 70 min, a delay of 62 min.
- (5) Irradiated *uvrA* (5 J/m²) = 23 min, a delay of 15 min.

In 8 min, the nonirradiated wild type should replicate ~960 kb of DNA (480 kb per fork). In the irradiated cells, replication forks would encounter ~107 pyrimidine dimers after 12 J/m², or 45 dimers after 5 J/m², if they replicated the same length of DNA. From the delay in [³H]thymidine incorporation, we can estimate the average delay per dimer (to the nearest second):

- (1) Wild type = 5 sec.
- (2) *uvrA* = 20 sec.
- (3) *uvrA dnaA46* = 35 sec.
- (4) *uvrA* (5 J/m²) = 19 sec.

These calculations demonstrate that (1) UV lesions delay fork progression; (2) by removing lesions, the excision repair system promotes fork progression; and (3) a significant amount of the DNA synthesis detected after UV irradiation is due to origin firing. Given there is also substantial UV-induced, DnaA-independent synthesis, these calculations reinforce the dangers of averaging the delay over the number of lesions and of ignoring the initiation of new synthesis. Not taking these factors into account can lead to the conclusion that forks skip over lesions without much delay.

The absolute delay of 35 min seen in the *uvrA* strain, or 62 min if origin firing is blocked, is in line with the prolonged delay

in replication past a leading strand lesion observed by Pages and Fuchs (2003). The delayed BrdU incorporation and delayed replication of the terminus region we observed in a UV-irradiated *uvrA* strain can be explained, therefore, if replication stops or slows down dramatically very soon after encountering a lesion in the leading strand template. However, since these cells have multiple lesions, we cannot exclude the possibility that forks skip over a few such lesions, as suggested (Heller and Marians 2006).

Acknowledgments

We thank those who kindly sent strains, Tim Moore and Andrew Savory for help with the microscopy, and Carol Buckman, Lynda Harris, and Lee Shunburne for excellent technical help. This work was funded by the Medical Research Council.

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Genes Dev. 2007, **21**:

Access the most recent version at doi:[10.1101/gad.417607](https://doi.org/10.1101/gad.417607)

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