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[25] Monitoring DNA Replication Following UV-Induced Damage in *Escherichia coli*

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Abstract

The question of how the replication machinery accurately copies the genomic template in the presence of DNA damage has been intensely studied for more than forty years. A large number of genes has been characterized that, when mutated, are known to impair the ability of the cell to replicate in the presence of DNA damage. This chapter describes three techniques that can be used to monitor the progression, degradation, and structural properties of replication forks following UV-induced DNA damage in *Escherichia coli*.

Introduction

The failure to accurately replicate the genomic template in the presence of DNA damage, whether spontaneous or induced, is thought to produce most of the genetic instability and mutagenesis observed in cells of all

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types. DNA damage encountered during replication produces genomic rearrangements when it resumes from the wrong place, mutagenesis when the incorrect base is incorporated opposite to the lesion, or even cell death when the block to replication cannot be overcome. Several genetic disorders clearly demonstrate the severe consequences that occur when damaged templates are inappropriately processed during replication. Cells from patients with classical xeroderma pigmentosum (XP) exhibit high frequencies of chromosomal rearrangements, mutagenesis, and lethality due to an inability to repair DNA lesions that block replication, rendering patients extremely sensitive to UV and prone to skin cancers (Cleaver *et al*., 1975, 1999; De Weerd-Kastelein *et al*., 1977; Tsujimura *et al*., 1990). These same phenotypes are also observed in the variant form of xeroderma pigmentosum (XPV) but are instead produced specifically by the loss of a polymerase that replicates through blocking DNA lesions (Cordeiro-Stone *et al*., 1997; Griffiths and Ling, 1991; Lehmann and Kirk-Bell, 1978; Masutani *et al*., 1999; Svoboda *et al*., 1998). Abnormal replication patterns and high rates of chromosomal exchanges are also observed in cells from Bloom's syndrome and Werner's syndrome patients, other genetic disorders characterized by cancer predisposition and premature aging, and can be traced to the loss of a RecQ-like DNA helicase (Ellis *et al*., 1995; Epstein and Motulsky, 1996; Fukuchi *et al*., 1989; Giannelli *et al*., 1977; Gray *et al*., 1997; Hanaoka *et al*., 1985; Karow *et al*., 2000; Kuhn and Therman, 1986; Langlois *et al*., 1989; Lonn *et al*., 1990; Mamada *et al*., 1989; Shiraishi, 1990; Yamagata *et al*., 1998). In *E. coli*, RecQ processes the nascent DNA at UV-induced blocked replication forks prior to their resumption and is needed to suppress illegitimate recombination (Courcelle and Hanawalt, 1999; Courcelle *et al*., 2003; Hanada *et al*., 1997). These genetic disorders clearly indicate that inaccurate replication in the presence of DNA damage contributes significantly to the incidence of cancer and aging in humans. Considering the severe consequences that result from the improper processing of damaged DNA, the molecular events that normally allow replication to accurately duplicate damaged genomic templates have been intensely studied over the years. This has resulted in the identification of a large number of candidate genes in both prokaryotes and eukaryotes which, when mutated, are known to impair the accuracy of replication in the presence of DNA damage. A remaining challenge has been to determine the precise roles that these gene products play in the recovery process. The DNA replication machinery and its associated proteins, like RecQ, are highly conserved among evolutionary diverged organisms, making *E. coli* an extremely valuable and appropriate system for dissecting the mechanism by which replication recovers from DNA damage.

In this chapter, we describe three cellular assays that are designed to help monitor and elucidate the events that occur at replication forks that encounter DNA damage. Each assay is designed to focus on a different aspect of replication following UV irradiation, and we try to discuss the advantages and shortcomings of each approach. The first assay measures the rate of DNA synthesis, the second measures degradation that occurs at the replication fork, and the third examines the structural properties of the replication fork DNA. We typically have utilized UV-induced DNA damage as our model lesion, but these methods should be adaptable to other forms of DNA damage or treatments that disrupt the replication machinery.

Description of the Methods and Technical Comments

General Considerations for Cell Culture and UV Irradiation

The parental strain utilized most frequently in our lab has been SR108, a *thyA deoC* derivative of W3110 (Mellon and Hanawalt, 1989). However, other backgrounds that contain *thy deo* mutations have also been used. When working with thymine auxotrophs, we always add 10 μg/ml thymine to all growth media and plates. Lower concentrations of thymine can result in impaired growth and cell filamentation (unpublished observations). The *thy* mutation is required for labeling with thymine, which in our hands gives a linear incorporation during long labeling periods. Thymidine in our media works efficiently for short (pulse) labeling periods, but is not incorporated linearly over extended time periods (Ann Ganesan, personal communication and unpublished observations).

For all these assays, frozen cultures are typically struck on a fresh Luria Bertani (LB) plate, supplemented with 10 μg/ml thymine, and incubated overnight at 37°. The following day, a single colony is used to inoculate 2 ml of Difco Minimal Davis Broth, a phosphate-buffered minimal medium, supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy medium). Davis broth contains 7 g dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate, and 1 g ammonium sulfate per liter of water, pH 7.0. Cultures are then grown overnight at 37° with vigorous shaking in test tubes with loose-fitting lids to allow adequate gas exchange.

UV-irradiation experiments are carried out under yellow lighting (>500 nm) or in the dark to prevent photoreactivation of cyclobutane pyrimidine dimers (CPD) by the enzyme photolyase. For yellow lights, we use F40/GO 40W Gold from GE. For UV irradiation, we use a Sylvania 15-watt germicidal lamp, which emits primarily 254-nm light. During UV

irradiation, care should be taken to ensure that the culture is agitated and that the irradiation time is sufficient to provide for a uniform exposure of the entire culture. The incident fluence should be determined with a UV photometer prior to use and the effective dose (CPD induced per kb of DNA) can be determined using T4 endonuclease V as previously described (Mellon and Hanawalt, 1989).

Measurement of DNA Synthesis Following UV-Induced Damage

The progress of the replication fork is impeded by UV-induced DNA damage. Over the years, several assays have been developed to monitor replication fork progression in the presence of DNA damage. The assay described here is a modification of one originally described by Khidhir *et al.* (1985). The protocol utilizes a dual, [^{14}C]-thymine and [^{3}H]-thymidine label to simultaneously monitor the overall DNA accumulation and the rate of DNA synthesis at specific times in thymine auxotrophs of *E. coli*.

To begin the assay, dilute the overnight culture 1:100 in 50-ml DGCthy medium supplemented with 0.1 μCi/ml [2-^{14}C]thymine, 50–60 mCi/mmol (Moravek Biochemicals, Brea, CA). Grow cells in a 37° water bath with vigorous shaking, monitoring cell growth using absorbance at 600 nm.

Once cells have reached an OD_{600} of precisely 0.3, begin sample collection as described later. When cultures above an OD_{600} of 0.3 are used, we observe that the rate of DNA synthesis begins to decrease before the 90-min time course ends, presumably because the culture begins to exit log-phase growth. Comparatively, cell densities that are significantly below 0.3 give highly variable results from experiment to experiment if not all the cells in the culture have entered log-phase growth.

UV Treatment and Recovery Assay

Pulse label solution:

Mix 2.97 ml DGCthy medium
30 μl [methyl-^{3}H]-Thymidine, 1 mCi/ml, 78 Ci/mmol stock (MP Biomedicals, Irvine, CA)

As the culture approaches OD_{600} of 0.3, pipet 50 μl of the pulse label solution into 60 5-ml polypropylene, round-bottom tubes. A typical time course involves 15 time points, taken in duplicate, and always includes both UV- and mock-irradiated treatments that are run in parallel.

Beginning 20 min before irradiation (time = −20 min), remove duplicate 0.5-ml aliquots of culture and add each aliquot to a tube containing the pulse label solution. Vortex the tubes for 2 s to mix the culture and ^{3}H label together and immediately place them into a 37° shaking water bath. After exactly 2 min, remove the tubes from the water bath and add ice-cold 5%

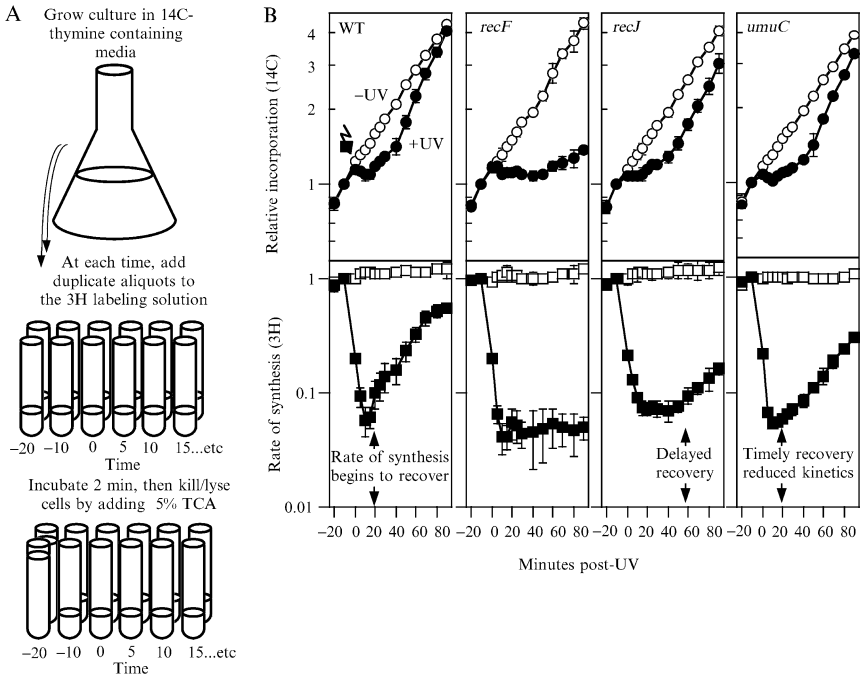

FIG. 1. DNA synthesis following UV irradiation. (A) Schematic of the radiolabeling and sampling process used in this assay. The steps involved in the technique are described in the text. (B) The rate of DNA synthesis and total DNA accumulation was measured for wild-type cells, *recF*, *recJ*, and *umuC* mutants at the times indicated following either UV irradiation (filled symbols) or mock treatment (open symbols). The relative amount of total DNA, ^{14}C (○), and DNA synthesis/2min, ^{3}H (□), is plotted. Typical initial values for ^{3}H and ^{14}C are between 7000–12,000 and 1500–2500 cpm, respectively, for all experiments.

trichloroacetic acid (TCA) to fill each tube. Figure 1A outlines the process used for sample collection. Repeat sampling process at time = −10 min. These two time points prior to treatment ensure that the cells are dividing and growing appropriately and provide a baseline measurement for DNA accumulation and DNA synthesis.

For UV treatment at time = 0, place 22 ml of the culture in a flat-bottomed container (e.g., the top of a plastic 90-mm Petri dish) and UV irradiate the culture with gentle agitation for the amount of time required for the desired dose. We typically irradiate at an incident dose of 1 J/m^2/s and expose the culture to 30 J/m^2. This dose induces a strong SOS response and generates approximately 1 CPD lesion per 8 kb DNA (Mellon and Hanawalt, 1989), but does not significantly reduce the survival of our

parental strain. Immediately after irradiation, transfer the culture to a fresh flask, prewarmed to 37°, and immediately add duplicate 0.5-ml aliquots of the culture to each of two tubes containing the pulse label, vortex, incubate at 37° for 2 min, and add ice-cold 5% TCA as before.

For the mock-treated sample, place 22 ml of the culture in a flat-bottomed container and gently agitate it without irradiation for the same amount of time as used in the UV-treated culture. Transfer mock-irradiated cells to a fresh flask, prewarmed to 37°, and immediately remove duplicate 0.5-ml aliquots of culture for pulse labeling as described previously. We have found that staggering the UV- and mock-treatment time course by 2 min facilitates accurate and timely sampling of cultures.

Continue to incubate both UV- and mock-treated cultures in a shaking 37° water bath for the reminder of the time course. At 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 min post-treatment, remove duplicate 0.5-ml aliquots of culture and add them to tubes containing the pulse label solution as before. At the end of each 2-min pulse period, add ice-cold 5% TCA to fill the tube. Keep collected samples at 4° until all time points have been taken.

Sample Preparation and Analysis of Recovery. The addition of 5% TCA serves to lyse the cells and precipitate DNA fragments longer than ~12 bp. To collect the acid-precipitable DNA from each sample and determine how much ^{3}H and ^{14}C was incorporated into each sample, we filter the samples through a vacuum manifold onto Whatman glass fiber filters. The empty sample tubes are then filled with 95% ethanol and again poured over each respective glass fiber filter to wash the remaining traces of precipitate from the tube and wash through any TCA that remains on the filter. The washed glass fiber filters are then rinsed a second time with 95% ethanol and the filters are allowed to dry in a 55° incubator (2 h is more than sufficient). Each filter is then placed into a scintillation vial, scintillation fluid is added, and the amount of radioactivity in each sample is determined. The windows or program on the scintillation counter must be set to exclude any overlap between the ^{3}H- and the ^{14}C-detection profiles.

Average the ^{3}H and ^{14}C counts from duplicate sample time points. Then, using the counts from the time = −10 min sample as a reference, determine the relative rate of DNA synthesis (based on ^{3}H counts) and total amount of DNA accumulation (based on ^{14}C counts) for each time point and treatment.

$$\text{Relative } {}^{3}\text{H at time X} = {}^{3}\text{H}_{\text{time X}}/{}^{3}\text{H}_{\text{time}-10}$$
$$\text{Relative } {}^{14}\text{C at time X} = {}^{14}\text{C}_{\text{time X}}/{}^{14}\text{C}_{\text{time}-10}$$

Plot the relative rate of DNA synthesis for UV- and mock-irradiated samples over time, and the total amount of DNA accumulation for UV- and mock-treated samples over time. In the case of mock-treated cultures, the ^{14}C-DNA continues to increase while the relative rate of DNA synthesis (^{3}H-DNA/2min) should either remain constant, or increase slightly, over the time course (Fig. 1B). Use of the dual label and mock-irradiation treatment provides several levels of internal controls that should help indicate the quality of the experimental data that is obtained. Significant fluctuations between time points in the amount of ^{14}C labeled-DNA could be indicative of pipetting errors or problems with filtering the samples. Large fluctuations in the ^{3}H-labeled DNA of mock-irradiated samples may also suggest problems in sampling or culturing techniques, and care should be taken when interpreting these results. A significant decrease in the rate of synthesis in mock-irradiated cultures suggests that the culture growth began to decrease before the end of the experiment.

Typical results for UV-irradiated wild-type cultures should produce a marked decrease in the relative rate of DNA synthesis immediately following the induction of DNA damage. After a 30 J/m^2 dose, we normally observe a drop in rate of about 90%. It is not clear what the remaining 10% of DNA synthesis reflects. *uvrA* mutants exhibit a similar decrease, suggesting that the synthesis does not represent residual repair replication. *dnaB*^{ts} mutants also exhibit a similar decrease when shifted to the restrictive temperature suggesting that it does not represent continued synthesis by the holoenzyme at a reduced rate (not shown). It is possible that this may represent radiolabeled nucleotides that are bound to proteins or lipids in our samples, which precipitate upon the addition of the TCA. The time at which the ^{3}H-DNA first begins to increase after UV irradiation is the time that we interpret DNA synthesis to begin to recover. For our parental or wild-type strains, this occurs between the 15- and 20-min time point after a 30 J/m^2 dose (Fig. 1B). The efficiency of replication recovery following UV-induced DNA damage can be established from the slope of the graphs.

In the majority of cases, the graph of total DNA accumulation reflects the trends observed for DNA synthesis rates, with a period of little to no DNA accumulation corresponding to times prior to when the rate of synthesis begins to recover. As shown in Fig. 1B, mutants can be identified by this assay that exhibit a failure to recover, a delayed recovery, and timely recovery but with reduced kinetics.

DNA Degradation at Arrested Replication Forks

Arrested replication forks are subject to enzymatic processing and degradation. To help characterize the enzymes that may process arrested replication forks, we developed the simple assay described later to examine

the degradation that occurs at the replication fork following arrest. In previous work this assay was used to show that following UV-irradiation, the nascent DNA of the replication fork is maintained and protected by the RecF-O-R proteins and partially degraded by RecQ, a 3′-5′ helicase, and RecJ, a 5′ single-strand nuclease (Courcelle and Hanawalt, 1999; Courcelle *et al*., 1997, 1999, 2003). Our recent experiments suggest that the nucleases and helicases which process the ends of the replication fork depend upon the nature of the impediment that blocked or disrupted the replication fork.

Dilute a fresh overnight culture 1:100 in 10-ml DGCthy medium supplemented with 0.1 μCi/ml [^{14}C]thymine. A 50-ml conical tube works well for growing this volume of culture. Grow cells at 37° with vigorous shaking, monitoring cell growth using absorbance at 600 nm.

While the culture is growing, prewarm 10-ml nonradioactive DGCthy medium in a 50-ml tube along with an empty 50-ml tube to 37°. In addition, set up 23 5-ml polypropylene, round-bottom tubes for sample collection, two for every time point, except for time 0, which serves as a reference for all other time points and is collected in triplicate.

UV Treatment and Degradation Assay

1× NET rinse buffer:
100 m*M* NaCl
10 m*M* EDTA, pH 8
10 m*M* Tris-HCl, pH 8.0

Prior to UV irradiation, the room should be set up to work under yellow light conditions as before. When the culture reaches an OD_{600} of 0.4, prefill three of the 5-ml polypropylene collection tubes with ice-cold 5% TCA, set up a vacuum filter holder and flask with a 0.45-μm general filtration membrane, and turn on the vacuum. Add 1 μCi/ml [^{3}H]thymidine to the culture for a 10-s pulse. Then, collect the cells by pouring the culture onto the 0.45-μm pore membrane filter. Once all the liquid has been sucked through, rinse the filter twice with 3-ml cold 1× NET. The EDTA in the NET buffer makes the cells more permeable and allows more of the [^{3}H] thymidine in the pulse label to be washed away. Figure 2A depicts the differential labeling of total and nascent DNA with ^{14}C and ^{3}H, respectively, resulting from this method.

Immediately resuspend the cells in the prewarmed, nonradioactive DGCthy medium by placing the filter into the conical tube and vortexing for about 5 s. Then, pour the culture into a flat-bottomed container and UV irradiate with gentle shaking for the desired dose, 30 J/m^2 in the example shown. After irradiation, transfer the culture to a fresh, warmed 50-ml

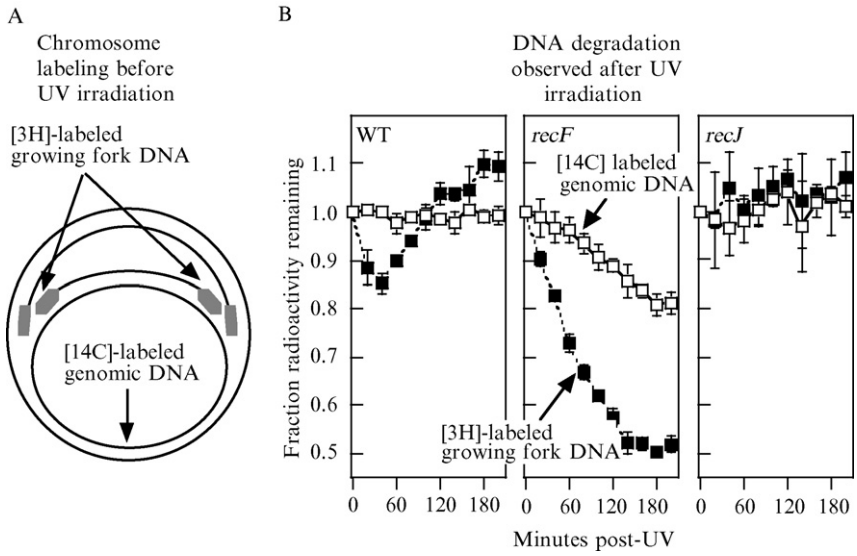

FIG. 2. Measurement of the amount of DNA degradation following UV-induced DNA damage. (A) Schematic depicting the differential labeling of total and newly synthesized DNA with [^{14}C]thymine and [^{3}H]thymidine, respectively. (B) The fraction of radioactive nucleotides remaining in the DNA from wild-type, *recF*, and *recJ* cells is plotted over time. Typical initial values for ^{3}H and ^{14}C are between 2500–4000 and 1200–1700 cpm, respectively, for all experiments. Total DNA (^{14}C, □); nascent DNA (^{3}H, ■).

tube. Remove triplicate 0.2-ml aliquots of the irradiated culture and place each aliquot in a tube with ice-cold 5% TCA (time = 0). Clearly, consistency and timing are important for this experiment when comparing the relative amount of degradation between strains. We find that about 20 s is required for rinsing and resuspending cells prior to irradiation, and with our UV apparatus, a 30 J/m^2 dose is delivered in 30 s. Therefore, to ensure consistency in the reference time point between strains and experiments, we typically remove the time = 0 aliquot 60 s after the time at which UV irradiation began.

Incubate the irradiated culture at 37° with vigorous shaking. Immediately before taking each time point, fill two tubes with ice-cold 5% TCA. Remove duplicate 0.2-ml aliquots of culture and place each into a tube with ice-cold 5% TCA at 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 min postirradiation.

Sample Preparation and Analysis of Degradation. Once the last time point is taken, collect the acid-precipitable DNA from each sample onto Whatman glass fiber filters and determine how much ^{3}H and ^{14}C was

incorporated into each sample as described in the previous assay. Average the ^{3}H and ^{14}C counts that are obtained from the duplicate (triplicate in the case of time = 0) time points. Then, using the counts from the time = 0 min sample as a reference, determine the relative amount of nascent DNA (based on ^{3}H counts) and total DNA (based on ^{14}C counts) that remains at each time. The loss of radioactivity represents the amount of degradation in total DNA and DNA made at replication forks immediately prior to UV irradiation.

$$\text{Relative } {}^{3}\text{H at time X} = {}^{3}\text{H}_{\text{time X}}/{}^{3}\text{H}_{\text{time 0}}$$
$$\text{Relative } {}^{14}\text{C at time X} = {}^{14}\text{C}_{\text{time X}}/{}^{14}\text{C}_{\text{time 0}}$$

Plot the relative amount of nascent DNA and total DNA remaining after UV irradiation over time. In the case of the total DNA for our parental cultures, we typically see little to no variation in the amount of DNA remaining over the time course (Fig. 2B). The lack of degradation in the total DNA provides an internal control and serves as a baseline upon which to compare the amount of degradation that occurs specifically in the nascent DNA following UV irradiation. For our parental cells, we typically observe that 10–20% of the nascent DNA is degraded at times prior to when replication resumes. In cells that are able to recover replication, the observed degradation of the nascent DNA ceases at the time when replication resumes (Fig. 2B). However, in mutants that fail to resume DNA synthesis, the nascent DNA degradation continues and is much more extensive (Fig. 2B). Once robust replication resumes, the assay is no longer able to effectively detect degradation and an increase in acid-precipitable counts is sometimes observed in the nascent DNA, presumably due to the reincorporation of the remaining intracellular pools of radiolabeled nucleotides. As shown in Fig. 2B, mutants have been identified that exhibit more extensive degradation than wild-type cells, suggesting that they have a role in protecting the arrested fork. Alternatively, other mutants like RecJ exhibit less nascent DNA degradation than wild-type cells, suggesting it acts to degrade the DNA at the fork following arrest.

Plasmid Replication Intermediates Observed by 2D N/N Gel Analysis

Following UV irradiation, the structural properties of the DNA molecule can be observed on replicating plasmids in *E. coli* using two-dimensional (2D) agarose gel electrophoresis. The technique can be used to observe UV-induced intermediates associated with both recombination and replication following arrest (Courcelle *et al.*, 2003). We use a 2D agarose gel technique adapted almost directly from Friedman and Brewer (1995). However, the method for preparing total genomic DNA for this

analysis is somewhat unusual in that it does not involve any procedures to enrich the samples for single-stranded fragments, nor does it involve any DNA precipitations that are often used in purifying DNA for 2D gel analysis. We have noticed that some DNA structural intermediates are sensitive to ethanol precipitation while others appear to form with higher frequencies. Thus the procedure was developed with the idea of keeping the manipulations during lysis and purification to a minimum.

An overnight culture of cells previously transformed with the plasmid pBR322 is grown at 37° with vigorous shaking in 2-ml DGCthy medium supplemented with 100 μg/ml ampicillin to maintain the plasmid.

To begin the experiment, 200 μl of the overnight culture is pelleted for 30 s at 12,000*g* in a microfuge tube. The cell pellet is resuspended in 200-μl fresh DGCthy and used to inoculate 20-ml DGCthy medium. This step is necessary to remove the ampicillin from the media as this antibiotic absorbs light strongly in the UV region of the spectrum and can significantly reduce the effective dose of irradiation to the culture. Grow cells in a shaking incubator at 37° without antibiotic selection.

UV Irradiation and DNA Isolation

2× NET:
200 m*M* NaCl
20 m*M* EDTA, pH 8
20 m*M* Tris-HCl, pH 8.0

Lysis Buffer:
1 mg/ml lysozyme
0.2 mg/ml RNase A in
TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

6× gel loading dye:
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
30% (v/v) glycerol in H_2O

While cells are growing, warm an empty flask at 37°. Set up six 2-ml microfuge tubes with 0.75 ml of 2× NET in an ice bucket, one for each time point. Make up lysis buffer (150 μl for each time point to be taken) and store on ice.

Prior to irradiation, the room should be set up to work under yellow light conditions as before. Once cultures have reached an OD_{600} of 0.5, place the culture in a flat-bottomed container and UV irradiate with gentle agitation. For studies using the plasmid pBR322, we typically irradiate with 50 J/m^2 because this dose generates, on average, 1 lesion per plasmid yet greater than 90% of our parental cells still survive to form colonies at this dose. Following irradiation, transfer the culture to a fresh, prewarmed

flask. Then, immediately remove a 0.75-ml aliquot of the culture and place it into one of the prechilled tubes containing 2× NET (time = 0). The cold temperature and EDTA in the NET buffer effectively stop further replication and repair events from occurring.

Centrifuge the sample at 14,000 rpm for 90 s in a microfuge. Decant the supernatant taking care to remove all the liquid and resuspend the pellet in 150-μl lysis buffer. Store samples on ice for the duration of the time course.

Continue to incubate the irradiated culture in a 37° shaking incubator. At 15, 30, 45, 60, and 90 min, remove 0.75-ml aliquots of the culture and add them to the prechilled tubes containing the 2× NET buffer and process these samples as described for the initial time point.

After the last time point, place the tubes in a 37° water bath for 30 min to lyse the cells. Then, add 10-μl 10 mg/ml proteinase K and 10-μl 20% Sarkosyl to the samples, mix gently, and then incubate at 50° for 1 h. Samples should be clear after this incubation.

To minimize shearing of the DNA, pipetting in the following steps should be done gently using wide-bore micropipet tips (we typically enlarge the holes of standard tips by cutting the ends off with a razor blade). Add 2 volumes of phenol to samples and mix for 5 min on an orbital platform. Then add 2 volumes of chloroform/isoamyl alcohol (24/1) and mix gently by inversion. Extract the aqueous phase and repeat the extraction with 4 volumes of phenol/chloroform/isoamyl alcohol (25/24/1) if necessary. Finally, extract the samples once with 4 volumes chloroform/isoamyl alcohol (24/1).

The samples are then dialyzed for 3 h on Whatman 0.05-μm pore disks that float in a 250-ml beaker filled with 2 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0. Note, with careful pipetting, each disk can support up to three 150-μl aliquots of sample. Larger volumes than this will cause the disk to sink. Cover the beaker with plastic wrap to prevent excessive evaporation.

Following dialysis, the DNA samples are digested with the desired restriction enzyme, in this example PvuII was used, following the manufacturer's instructions. We typically make up a 5× master mix of the enzyme in the buffer, then add 20 μl of the enzyme mix to 80 μl of each genomic DNA sample. PvuII restricts pBR322 once just downstream of the origin, however the choice of restriction enzyme to use will depend on the plasmid being examined and the type of structural information being sought. A more thorough discussion of this and other aspects of neutral/neutral 2D gel electrophoresis can be found elsewhere (Brewer and Fangman, 1987; Dijkwel and Hamlin, 1997).

Following digestion, add 1 volume of chloroform/isoamyl alcohol (24/1) to the samples to denature the restriction enzyme prior to electrophoresis and then add 20 μl of 6× gel loading dye directly to each sample. Since no

precipitation steps are involved, and the cell pellets from each sample are resuspended and lysed in an equal volume, the DNA concentration in each sample is typically consistent throughout the time course and yields about 20 ng/μl of genomic DNA.

Two-Dimensional Agarose Gel Electrophoresis

10× TBE: Per liter, dissolve
108 g Tris base
55 g boric acid
40 ml 0.5 *M* EDTA (pH 8.0)
add H_2O to 1 liter

Cast a 200-ml 0.4% agarose gel, 1× TBE in a 13 × 17-cm tray using a 20-well comb (approximate capacity 40 μl). Once gel is solidified at room temperature, pour 1× TBE buffer over the gel. Remove comb gently and fill the electrophoresis rig with enough 1× TBE to submerge the gel completely.

Load a lambda-Hind III size marker in the first lane, then skipping lanes, load 30 μl of each DNA sample into the wells. Loading every other lane makes it easier to cleanly cut the gel lanes for casting in the second-dimension gel. Extra gel lanes may be used to load a second set of samples to check the quality and quantity of the DNA in your samples. Run the gel at 1 V/cm until the (lower) bromophenol blue marker dye has migrated 6.5 cm (~12–14-h in our gel rigs). Note, the gel may be run longer depending on the time points and size of your gel in the second dimension. This migration distance was selected because it yields sufficient resolution and allows us to fit six samples on our gel for the second dimension.

For the second dimension, prepare a 500-ml 1% agarose gel solution in 1× TBE and allow it to cool to between 45 and 50°.

Slice the gel evenly between lanes. We use a large butcher knife to cut our gels, which we find makes it easy to slice even segments. The first lane containing the size markers can be stained with ethidium bromide and visualized. If a second set of lanes was loaded, these too can be stained and visualized. Once the migration distance of your linear fragment has been determined, trim the gel lanes to a length that comfortably spans this size. Under the gel conditions described here, linear pBR322 runs above the bromophenol blue marker. Rotate each gel lane 90° and place the first three lanes lengthwise along the top of a leveled 20 × 25-cm electrophoresis tray. The next three lanes are then placed across the middle of the gel tray. Pipette a small amount of the 1% agarose gel solution around each gel slice to set it in place. This small amount of agarose should set in 1–2 min. Then, pour the remaining 1% agarose solution into the gel tray, allowing it

to hit the surface of the tray first and taking care to pour a smooth, even layer. The agar should completely cover the sliced lanes from the previous gel. If the agarose solution is too hot when it is poured, it could partially melt the agarose slices from the first dimension or affect fragile DNA structures.

Once the gel has solidified, place it into an electrophoresis rig and fill with enough 1× TBE to submerge the gel. Run the second-dimension gel at 6.5 V/cm until the xylene cyanol dye front has migrated about 10.5 cm (~7 h in our gel rigs).

After electrophoresis, transfer the DNA to a positively charged nylon membrane (e.g., Hybond N+) using standard procedures (Spivak and Hanawalt, 1995).

Prepare a ^{32}P-labeled pBR322 probe using nick translation. We use the protocol included with the nick translation kit from Roche with good success.

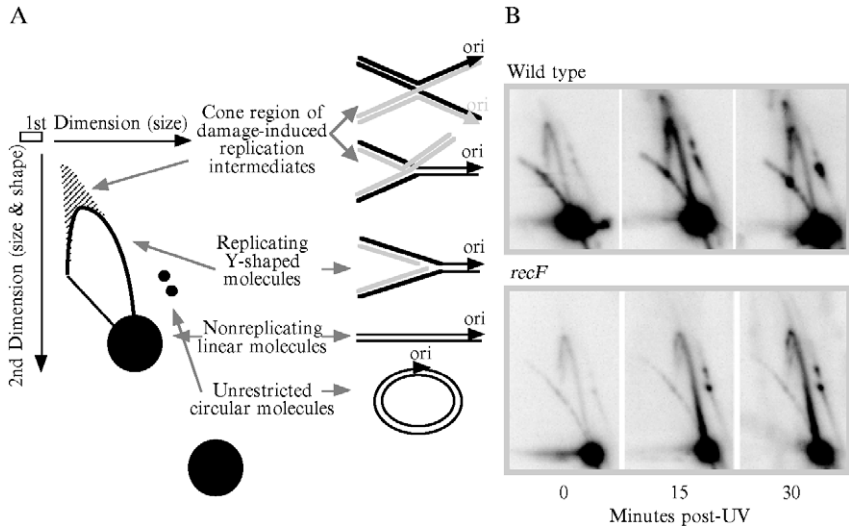

FIG. 3. UV-induced replication intermediates observed by neutral/neutral two-dimensional gel electrophoresis. (A) Predicted migration pattern for PvuII-digested pBR322 plasmid after UV treatment using 2D analysis. Nonreplicating plasmids run as a linear 4.4-kb fragment. Normal replicating plasmids form Y-shaped structures and migrate more slowly due to their increased size and nonlinear shape, moving as an arc that extends from the linear fragment. Double Y- and X-shaped intermediates migrate in the cone region. (B) Blocked replication fork and cone region molecules accumulate transiently in wild-type cells after UV irradiation. RecF mutants do not accumulate cone region intermediates.

Hybridize the membrane with the radiolabeled probe and visualize and quantitate the radioactivity on a phosphorimager. The predicted migration pattern for PvuII-linearized pBR322 is shown in Fig. 3A. It should be noted that this method can be easily adapted to examine other plasmids or used with alternative restriction enzymes. In wild-type cells following UV-induced damage, we typically see a transient increase in the amount of replicating Y-shaped intermediates due to an accumulation of blocked replication forks at UV lesions (Fig. 3B). In addition, double Y- or X-shaped intermediates also accumulate transiently in the cone region, peaking around 30 min following UV irradiation before waning at a time that correlates with the repair of the DNA lesions and the recovery of robust replication (Courcelle *et al*., 2003). In contrast, cone region intermediates are not observed in *recF* mutants.

Taken together, the three assays described previously can be applied to various *E. coli* mutants to help characterize the potential functional role of those gene products *in vivo*. In the case of *recF* we believe that these assays are consistent with the idea that RecF is needed to protect and maintain the structural integrity of replication forks arrested at UV-induced damage.

Concluding Remarks

The three assays described previously each focus on a different aspect of the replication fork following DNA damage. It is critical to keep in mind, however, that while the first assay is designed to quantify the amount of DNA synthesis at the fork and the second to measure DNA degradation, both processes are clearly occurring simultaneously in the cell. While the use of both assays provides a more comprehensive picture of the events occurring at the replication fork, each process is likely to partially interfere with the measurement of the other. For instance, if significant amounts of degradation are occurring in the nascent DNA, then the observed amount of newly DNA synthesized in our assay may be less than that which is actually occurring. The limitations of these assays and determining precisely what they can measure are important factors that should be considered when interpreting these assays and when trying to develop new methods to observe and tease apart the biochemical reactions that occur in living cells.

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