Video Article

Visualization of UV-induced Replication Intermediates in *E. coli* using Two-dimensional Agarose-gel Analysis

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Abstract

Inaccurate replication in the presence of DNA damage is responsible for the majority of cellular rearrangements and mutagenesis observed in all cell types and is widely believed to be directly associated with the development of cancer in humans. DNA damage, such as that induced by UV irradiation, severely impairs the ability of replication to duplicate the genomic template accurately. A number of gene products have been identified that are required when replication encounters DNA lesions in the template. However, a remaining challenge has been to determine how these proteins process lesions during replication *in vivo*. Using Escherichia coli as a model system, we describe a procedure in which two-dimensional agarose-gel analysis can be used to identify the structural intermediates that arise on replicating plasmids *in vivo* following UV-induced DNA damage. This procedure has been used to demonstrate that replication forks blocked by UV-induced damage undergo a transient reversal that is stabilized by RecA and several gene products associated with the RecF pathway. The technique demonstrates that these replication intermediates are maintained until a time that correlates with the removal of the lesions by nucleotide excision repair and replication resumes.

Protocol

1. Growth and UV Irradiation.

1. 200µl of a fresh overnight culture containing the plasmid pBR322 grown in Davis medium[1] supplemented with 0.4% glucose, 0.2% casamino acids, and 10 µg/ml thymine (DGCthy medium) and 100 µg/ml ampicillin is pelleted. The cell pellet is then resuspended in 200µl DGCthy medium lacking ampicillin and used to inoculate 20 ml of DGCthy medium.
2. Cultures are grown without ampicillin selection in a shaking incubator at 37° C to an OD_{600} of 0.5 (~ 5 x 10^8 cells/ml). Growth without ampicillin avoids selection against abnormal or unproductive replication intermediates that may arise in some mutants. In addition, if using UV light to induce damage, the removal of the ampicillin from the media is necessary because it absorbs strongly at these wavelengths and shields the cells, reducing the effective dose of UV.
3. Working under yellow lights, the culture is placed in a 15cm diameter Petri dish on a rotating platform for agitation. Our cultures are placed at a distance from a 15-watt germicidal lamp that produces an exposure rate of ~1 J/m2/sec, which is measured using a UVC photometer. Cultures are irradiated with 50 J/m2 and then placed immediately back into the shaking 37° C incubator for the duration of the experiment. This dose produces, on average, 1 cyclobutane pyrimidine dimer every 4.5 kb of ssDNA . The yellow lighting prevents photoreactivation-reversal of cyclobutane pyrimidine dimers by photolyase.

2. DNA Isolation.

1. At times when replication intermediates are to be examined, a 0.75 ml aliquot of the culture is placed into 0.75 ml ice cold NET30 Buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 30 mM EDTA) and placed on ice until the end of the time course. We typically run a 90 minute time course, with samples examined at 0, 15, 30, 45, 60, and 90 minutes. The EDTA and cold temperature serve to effectively stop replication and nucleotide excision repair.
2. Each sample is then pelleted, resuspended in 150 µl of 1.5 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10 mM Tris, pH 8.0, 1 mM EDTA), and lysed at 37°C for 20 min. At this time, 10µl of proteinase K (10mg/ml) and 10µl of 20% sarkosyl are added and the incubation is allowed to continue for 1 hr. Proteinase K and sarkosyl help to release DNA fragments that may be associated with the membrane or proteins before phenol extraction occurs. Since actively replicating DNA is often bound to protein or membrane complexes, this helps increase the yield of replication fragments that are recovered.
3. Samples are then extracted by adding 2 volumes of phenol to each sample and the tubes are gently inverted for 5 minutes. Then 2 volumes of chloroform/isoamyl alcohol (24/1) are added and the tubes are gently inverted again for 5 minutes.
4. The samples are centrifuged at 14,000 rpm in a microcentrifuge for 5 minutes and the top aqueous phase of each sample is removed, and placed into a fresh tube. Then 4 volumes of chloroform/isoamyl alcohol (24/1) are added, the tubes are gently inverted for 5 minutes, and centrifuged again at 14,000 rpm for 5 minutes.
5. The top, aqueous phase in each sample is then dialyzed for 1 hour by spotting 100µl of each sample on a 47mm Whatman 0.025 µm pore disk which floats on 250 ml of 0.1X TE in a beaker. Because replicating structures with single strand regions or branch points are more susceptible to shearing, we typically cut our pipette tips off with a razor blade to make the mouth wider and minimize shearing forces. In general, pipetting should be kept to a minimum until after the DNA has been digested with restriction enzymes.
6. Each sample is then digested with PvuII (New England Biolabs) which linearizes the plasmid just downstream from the origin of replication.
7. Prior to loading in the agarose gel, 100µl chloroform and 20µl of 6X loading dye that contains Bromophenol Blue and Xylene Cyanol are added to each sample and mixed. Then, 40µl of the aqueous phase containing the loading dye is loaded into the gel. To minimize structural biases, structural artifacts, and DNA shearing, this isolation procedure does not include any steps to enrich for single strand regions, precipitate, or concentrate the DNA samples following cell lysis.

3. 2D Gel and Southern Analysis.

1. The 2-dimensional agarose-gel analysis is modified from 3. For the 1ST dimension, the restricted DNA samples are run through a 0.4% agarose gel in 1X TBE at 1V/cm. One Liter of 10X TBE stock solution contains:
   - 108 g Tris Base
   - 55 g Boric Acid
   - 40 ml 0.5M EDTA (pH 8.0)

2. A lambda Hind III size marker is loaded in the first lane, then samples are loaded in every other lane. We typically run the first dimension ~12-15 hrs. Skipping lanes makes it easier to slice the lanes out to cast in the second dimension. The low voltage and low percent agarose gel serves to separate the DNA fragments primarily based upon size.
3. For the second dimension, gel lanes are sliced out of the first dimension gel using a large butcher's knife. The first lane containing the lambda Hind III marker can be stained with ethidium bromide. Using the lambda Hind III marker as a guide, crop and discard the region of the gel that is below where the linearized plasmid is expected to run. Under these conditions, we find that linearized pBR322 runs slightly above the xylene cyanol stain.
4. To cast the second dimension, each lane is placed horizontally across the top of an empty gel caster. A solution of 1.0% agarose in 1X TBE is prepared and cooled to 55°C. At this time, the gel solution is poured in to cast the second dimension, making sure to completely cover the gel slices. Once the gel has set, it is run at 6.5V/cm in an electrophoresis unit that allows the buffer to recirculate. We typically run the second dimension ~5.5-7 hrs. The high voltage and high percent agarose gel effectively separates the DNA fragments based on their shape, as well as size. Nonlinear shapes run more slowly through the second dimension.
5. Following electrophoresis, the gel is then rinsed in H_2O, washed twice in 400ml of 0.25M hydrochloric acid for 15 minutes, rinsed with H_2O;, and then washed twice in 400 ml 0.4M NaOH for 30 minutes. The acid washes serve to partially nick the DNA molecules into smaller fragments that transfer more efficiently. This step is necessary due to the large size and unusual shapes of the DNA replication intermediates.
6. The DNA in the gels is then transferred to a Hybond N+ nylon membrane [4]. We use a downward alkali transfer system with 0.4M NaOH. Briefly, two sheets of blotting paper soaked in 0.4M NaOH are placed on a large stack of paper towels. The nylon membrane is also soaked in 0.4M NaOH and placed on top of the blotting paper. The gel is then carefully layered on top of this, followed by another piece of blotting paper, wetted in the NaOH solution. Finally, a long piece of wetted blotting paper is layered across the top and its two ends are placed into a dishes containing 1 L of 0.4M NaOH solution to serve as a wick. We typically let the DNA transfer for 6-12 hrs.
7. The nylon membrane is removed, washed 20-30 sec in 5X SSC Buffer, and placed in a hybridization roller bottle containing 10.5ml Prehybridization Solution. Then, the membrane is incubated at 42°C with rotation for more than 6hrs. Prehybridization solution:
   - 5.0 ml formamide
   - 0.5 ml 20% SDS
   - 2.5 ml 20X SSC*
   - 2.0 ml 50X Denhardt's*
   - 0.5 ml Salmon Sperm DNA (10mg/ml)

   *recipes for SSC and Denhardt's can be found in 5

8. During the prehybridization period, 1 µg of pBR322 plasmid is labeled with 32P by nick translation according to the protocol supplied by Roche using alpha labeled [32-P]-dCTP. The radiolabeled probe (100µl) is denatured by incubating at 98° C for 10 minutes in a screw cap microcentrifuge tube, and then placed immediately on ice.
9. The denatured probe is added to 5.9 ml hybridization solution, which contains:
   - 3.0 ml formamide
   - 1.5 ml SSC
   - 1.0 ml H_2O
   - 0.15 ml 50X Denhardt's
   - 0.10 ml 20% SDS
   - 0.15 ml 10mg/ml Salmon Sperm DNA

10. The prehybridization solution is poured out of the roller bottle and the hybridization solution is poured in. The roller bottle is then returned to the 42° C incubator and rotated for more than 12hrs.
11. The hybridization solution is poured out of the roller bottle. Then, the blot is washed 4 times for 20 minutes in the roller bottle with ~150 ml of a wash solution containing 0.5X SSC, 0.1% SDS at 42° C with rotation.
12. Following the last wash, the membrane is placed on a paper towel until the liquid has visibly disappeared. The membrane is then wrapped in polyvinyl-chloride plastic wrap and exposed to a phosphorimager screen. We visualize and quantify the radioactivity using a Storm 840 and its associated ImageQuant Software.

4. Representative Results:

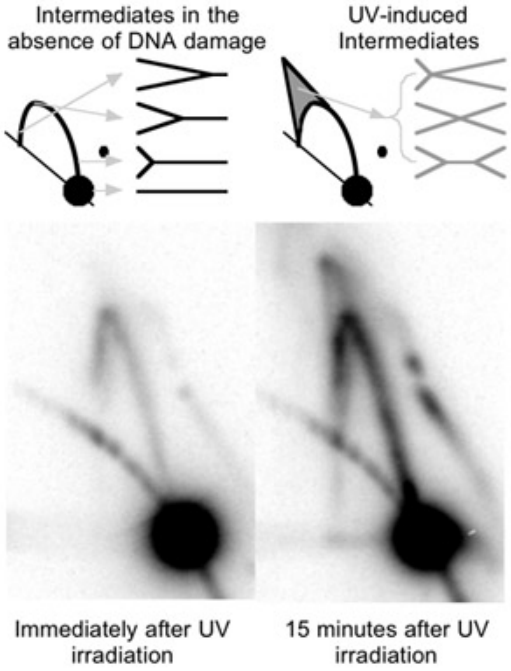

Figure 1. Plasmid replication intermediates observed in the presence and absence of UV-induced DNA damage. The migration pattern of PvuII digested pBR322 plasmid observed by 2D-agarose-gel analysis is diagrammed. Non-replicating linear plasmids run as a linear 4.4-kb fragment. Replicating plasmids form Y-shaped structures that migrate slower than the non-replicating fragments due to their larger size and nonlinear shape. This migration pattern forms an arc that extends out from the linear region towards the well. Following UV-irradiation, double-Y or X-shaped molecules are observed in the cone region that migrates more slowly than the arc of Y-shaped molecules. An example of the Southern analysis of the 2D gel probed with 32P-labeled pBR322 is shown for cells immediately after UV irradiation and 15 minutes after UV irradiation (reproduced with permission from the publisher).

Disclosures

No conflicts of interest declared.

Discussion

Typical results obtained from wild type cells in the presence and absence of UV-induced damage are shown in Figure 1. In the absence of damage, ~1% of the total plasmid DNA can be found in the Y arc when cells are rapidly growing in exponential phase. Following irradiation, a transient increase in Y shaped molecules is observed as blocked replication forks accumulate at damaged sites. The X-shaped replication intermediates also transiently accumulate and persist until a time that correlates with when the lesions are repaired.

In place of the Southern analysis, the replication intermediates can be punched out of the gel with a plastic drinking straw, purified, and observed directly by electron microscopy. We have used this approach successfully to identify gene products required to process replication forks that encounter DNA damage and to identify abnormal replication intermediates that accumulate in these mutants . In addition, this approach can be easily modified to examine other forms of DNA damage.

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