

### 3. Project Significance

Ultraviolet radiation, chemical carcinogens, and even the normal intermediates produced during oxidative respiration, all chemically react with the DNA in our cells in a way that damages the genetic information and can block DNA replication. It is clear that inaccurate replication of damaged DNA is responsible for the majority of cellular rearrangements and mutagenesis which are typically observed in all cancer cells. Additionally, several cancer prone genetic disorders exhibit defects in the replication of damaged DNA and attest to the severe consequences which result when this process is impaired (1).

Despite its importance in maintaining genomic stability, the fundamental mechanism by which replication blocking DNA damage is processed and repaired remains largely uncharacterized. A primary obstacle which has previously prevented this mechanism from being examined has been the lack of an assay for isolating the replicating fragments of DNA from the vast excess of nonreplicating DNA. In this proposal, we describe a novel assay, based upon two-dimensional gel analysis, to isolate these replicating DNA fragments directly for molecular characterization. Using this technique, we will be able to identify when replication is blocked by DNA damage and determine the mechanism by which the blocked replication forks subsequently recover faithful replication.

### 4. Background

A large number of genes have been identified in both prokaryotes and eukaryotes which, when mutated, impair the accuracy and processivity of replication in the presence of DNA damage (1). Considering the severe consequences which result from inaccurate replication, what are the molecular events which normally allow replication to faithfully duplicate the chromosome even when DNA damage is present? The fundamental mechanism by which DNA replicates is highly conserved among even the most evolutionarily diverged organisms, making *E. coli* an extremely valuable and appropriate system for dissecting the molecular mechanism by which replication recovers from DNA damage in all cells.

Irradiation of cells with near UV light induces lesions in the DNA which block replication. In *E. coli*, like in human cells, DNA replication is transiently inhibited following a moderate dose of UV irradiation, but it efficiently recovers following the repair of the UV-induced lesions by nucleotide excision repair enzymes (2). In mutants defective in nucleotide excision repair, the recovery of replication following UV-irradiation is severely impaired and results in a loss of semiconservative replication, high frequencies of chromosomal exchanges, and extensive cell death (2, 3). In contrast, normal cells efficiently suppress these recombination events and the survival and recovery of replication is greatly enhanced even following comparatively high doses of UV irradiation (2).

In addition to the repair of the DNA lesions, the recovery of replication also requires the function of RecA and RecF pathway proteins (4, 5). Both RecA and RecF have previously been characterized for their ability to bind and pair homologous strands of DNA, an activity which is thought to be critical during recombination (6). However, during genomic replication, this same enzymatic activity is also thought to be required to protect and maintain the strands of the replication forks when they are blocked by DNA

damage (5). In the absence of either RecA or RecF replication does not recover, the replication fork is not maintained, and extensive degradation of the nascent DNA occurs (5).

The recovery of replication in wild type cells appears to occur through a pathway involving both nucleotide excision repair and RecA pathway functions, since in the absence of either component cell survival and replication recovery do not occur (2). Yet, at present, the mechanism by which these genes function together to promote recovery has not been examined. One obvious possibility is that the DNA lesions which block the advancing replication forks are substrates for nucleotide excision repair. Thus, the role of the *rec* genes in recovery may be to simply maintain the strands of the replication fork until the lesion can be repaired. Alternatively, the *rec* proteins could be more intimately involved in repair, possibly being required to process or remove the replication machinery, such that the nucleotide excision repair machinery can access and repair the DNA lesion. A third possibility is that the *rec* genes function independently to recombine around the lesions. A slow process of recombination may then provide the time necessary for the remaining DNA lesions around the genome to be repaired before replication resumes.

Clearly, to differentiate between these possibilities, a method which allows for the isolation and characterization of DNA damage-blocked replication forks is required such that the mechanism by which replication recovers in the presence of DNA damage can be examined directly.

## **5. Objectives**

1. To isolate the replicating DNA fragments from UV-irradiated *E.coli* throughout the period of replication inhibition and recovery.
2. To determine the frequency that UV-induced lesions block DNA replication on the DNA template and characterize the structural intermediates which arise at blocked replication forks during the recovery process.

## **6. Project Plan**

1. To separate the replicating DNA fragments from the non-replicating fragments, I will take advantage of the unique structural properties of replicating DNA. Unlike nonreplicating DNA fragments, replicating DNA fragments are branched and migrate more slowly than linear fragments of the same sequence during agarose gel electrophoresis. I will adapt a standard two dimensional agarose gel analysis to isolate and examine replicating plasmid molecules in *E. coli*.

The isolation of the replicating plasmids from nonreplicating plasmids is performed as follows. Total genomic DNA is purified from an exponentially growing culture of *E.coli* containing the plasmid which is to be examined (*puc18*). The genomic DNA is then digested with a restriction enzyme which cuts the plasmid at a unique site (*Alw NI*). The digested genomic DNA is then run overnight in a standard 0.5% agarose gel which separates DNA molecules based upon size as shown (figure 1a) (7). Note that although all nonreplicating molecules will migrate as 3kb linear strands of DNA, replicating plasmid molecules will vary between 3kb and 6kb of DNA depending upon how much of the plasmid has replicated at the time of preparation and will migrate more slowly than the nonreplicating, linear molecules. To further separate the parental strands

of the replicating DNA from the nascent DNA strands, the gel can be rotated 90 degrees and run in alkali buffer to denature the parental and nascent strands as shown (figure 1b). The parental strands will migrate as full length 3kb fragments while the partially replicated, nascent strands will migrate as smaller DNA fragments.

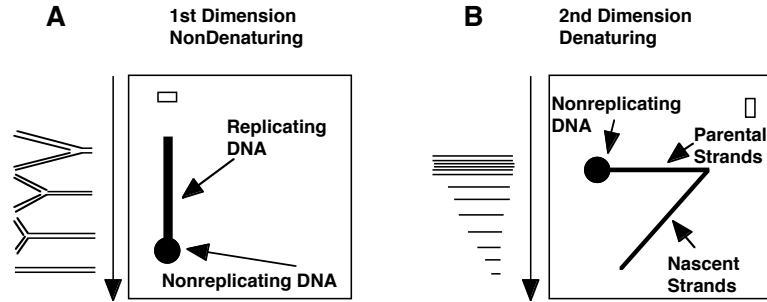


Figure 1. Separation of replicating DNA fragments from nonreplicating fragments by agarose gel electrophoresis.

Following separation in agarose gels, the gel is transferred to a Nylon membrane, and the quantity of replicating plasmid molecules will be detected by probing with  $^{32}\text{P}$ -labeled strand-specific probes (8).

In order to examine the recovery of replication forks blocked at DNA damage, this technique will be used to isolate replicating plasmid molecules from UV-irradiated cells. A UV dose of 40 J/m<sup>2</sup> produces, on average, one UV-induced lesion on each strand of a 6kb fragment (2, 8). We have previously shown that this dose inhibits replication for approximately 20-40 minutes (2). To confirm that replicating fragments blocked by UV-induced lesions can be isolated, purified genomic DNA from the irradiated cultures will be either treated or mock-treated with T4 Endonuclease V, an enzyme which cleaves DNA at sites of UV-induced lesions prior to agarose gel analysis (8). Since all DNA replication is inhibited at this dose, the DNA molecules migrating above the linear fragments should represent replicating fragments that are arrested at DNA lesions. Thus, treatment of the samples with T4 Endonuclease V is expected to cleave the replicating strands which contain a UV-induced lesion, manifested as a loss of full length replicating DNA fragments (because they were cleaved) in the treated samples compared to mock-treated samples (8).

A variety of conditions (i.e. growth phase of the cells, UV dose, high voltage vs. low voltage gel) will be examined to obtain the maximal separation and yield of replicating plasmid molecules during the first three months of the grant period.

2. The ability to isolate damage-blocked replicating fragments, as outlined above, will allow me to determine several, previously unknown, aspects of the mechanism by which replication recovers. For instance, it has been suggested that lesions in the leading strand template, but not the lagging strand template, may block chromosomal replication. If true, then by using strand-specific  $^{32}\text{P}$ -labeled riboprobes to detect each template strand of replicating fragments separately one would expect that, of all the blocked replicating fragments isolated, only leading strand fragments would contain UV-induced lesions, as measured by TEV treatment.

Additionally, some current models of replication recovery have speculated that replication forks “collapse” at DNA lesion to form double strand breaks, while others have proposed that the replication forks are maintained throughout the recovery process (9, 10). The assay described here, offers a direct test to differentiate between these possibilities. By isolating DNA from cells throughout the period of inhibition and

recovery, we can directly measure the quantity of replicating fragments which are maintained during the recovery process. If the recovery of replication occurs through a double strand break intermediate, the replicating fragment would become linearized and we should detect a loss of branched replicating molecules following UV irradiation. Alternatively, if the replication forks are maintained throughout the recovery process, the quantity of replicating molecules should remain constant.

An additional aspect which can now be addressed using this assay is to determine if the blocking DNA lesions are substrates for the nucleotide excision repair proteins. By measuring the frequency of T4 Endonuclease V sensitive sites in the replicating DNA fragments over time, we can directly examine whether the lesions present in the replicating fragments are being repaired.

## 7. Expected Results

Through the development of a novel assay to isolate replicating fragments blocked at DNA lesions, we will be able to determine the strand at which replication is blocked and the fundamental pathway by which replication faithfully recovers following DNA damage. Although this work focuses upon the recovery of replication on plasmid molecules, the technique can clearly be applied to examine and compare the recovery of replication directly on the *E. coli* chromosome. Importantly, our ability to isolate replication forks directly, combined with our ability to measure DNA repair, will provide the foundation for future studies to directly measure the repair rates and intermediates which arise in various excision repair and recombination deficient mutants. For instance, I have previously shown that *rec* mutants are able to repair DNA lesions in the bulk genomic DNA with kinetics similar to those in wild type cells (2). If however, one or several of the *rec* mutants exhibit defects in repairing the DNA lesions at replication forks, it would suggest that these proteins are needed to actively process the replication fork and participate in the repair process.

By determining the mechanism by which replication normally recovers faithfully, we will be able to identify the events which can lead to illegitimate recovery, genomic rearrangements, and lethality in the presence of DNA damage. Clearly, the faithful recovery of replication is critical to maintaining genomic stability in all cells and is of significant interest to the National Cancer Institute, as outlined in their desired aims and goals, making it a strong candidate for future funding through this agency.

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## 8. Qualifications of Principal Investigator

University of Vermont	BS	1992	Biology
Stanford University	Ph.D.	1999	Cancer Biology
University of Paris V	Post-Doc	2000	Mutagenesis
Mississippi State University	Asst Prof	2000	Bacterial Genetics, Biology

### Professional Experience

1989-1992. *Undergraduate Research, University of Vermont.* Research in the laboratory of Susan S. Wallace. Inducible responses to oxygen stress in *Escherichia coli*. Repair of DNA damage induced by oxygen free radicals. Characterization of mutagenic variability of DNA lesions based upon sequence context.

1992-1994. *Graduate Research, Stanford University.* Research in the laboratory of Dr. Michael Lieber. Generation of antigenic diversity in the immune system. Biochemical studies on immunoglobulin class switch recombination and purification of protein factors that target recombination to specific sites in the genome.

1994-1999. *Dissertation Research, Stanford University.* Research in the laboratory of Dr. Philip C. Hanawalt. DNA replication and repair in *Escherichia coli*. Replication recovery and DNA repair mechanisms following ultraviolet light induced DNA damage.

1999-2000. *Postdoctoral Research, University of Paris.* Research in the laboratory of Dr. Miroslav Radman. Characterization of the mechanism by which cells limit chromosomal replication to an exact doubling of their genetic material.

### Fellowships, Awards, and Other Professional Activities

- Chemical Rubber Company Chemistry Award*, undergraduate chemistry, UVM, 1990
- HELIX minigrant award*, undergraduate research grant, UVM, 1991
- Benedict Award in Biology*, outstanding biology graduate UVM, 1992
- Vermont-NEA Townsend Scholarship*, 1993
- National Science Foundation Fellowship*, 1993-1996
- Workshop. Histology of Cancer*, American Cancer Society, Keystone, CO, 1995.
- National Cancer Institute Traineeship, Cancer Biology Program*, 1996-1999
- Instructor, Stanford U.* 1997-1998. "DNA Replication", "DNA Repair Mechanisms"
- Graduate Admissions Committee.* 1998. Stanford U. Program in Cancer Biology.
- European Molecular Biology Organization (EMBO) Post-Doctoral Fellowship*, 1999-2000.
- Reviewer/Referee.* 1996-present. Manuscripts submitted to *Proc Nat Acad Sci USA*, *Genetics*, *Molec and Gen Genet*, *Cell*, *J Bact*.

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Courcelle J., Khodurski A., Peter B., Brown P.O., and Hanawalt P.C. Comparative gene expression profiles following UV exposure in wild type and SOS deficient *Escherichia coli*. (submitted *EMBO*).

Courcelle, C.T., Courcelle J., Prichard M.N., Hanawalt, P.C., and Mocarski E.S. Cytomegalovirus requires uracil-DNA glycosylase activity for transition to late phase viral DNA replication. (manuscript in preparation).