THE RECOVERY OF DNA REPLICATION IN *ESCHERICHIA COLI* IRRADIATED WITH ULTRAVIOLET LIGHT

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Abstract

The order of discovery can have a profound effect upon the way in which we think about a protein's function. Historically, because many Rec proteins were identified through recombination assays, functional studies on these proteins have focused primarily upon how they may promote the rearrangement of genetic material. Paradoxically however, during the cellular asexual reproductive cycle, many of these proteins are required to maintain the integrity of the genome rather than to rearrange it. Therefore, in order to understand the biological role that recombination plays in the cell, it is important to keep in mind the strategy and products of the reproductive cycle in which it is being studied.

Several genes in the *E.coli recF* pathway were isolated as mutations which conferred recombination deficiencies when the major pathway of recombination (the *recBCD* pathway) had been inactivated. In an otherwise wild type background, *recF* pathway mutants remained fully proficient in recombination although, surprisingly, many were hypersensitive to UV. That observation implies that the UV hypersensitivity in these mutants may not be due to an inability to carry out recombination. We therefore sought to understand the unique cellular role of *recF* that is required for resistance to UV-irradiation. In so doing, we found that the requirement for *recF* function correlated strongly with ongoing DNA replication. In the absence of *recF*, replication failed to recover following irradiation, and extensive degradation of the nascent DNA at the replication fork was observed. These and other data suggested that the UV hypersensitivity in *recF* mutants could be explained by an inability to maintain replication forks blocked by DNA damage. That idea is also consistent with genetic data suggesting that recombination mediated by *recF* may initiate from a recombinational intermediate which is structurally similar to a replication fork. However, during asexual reproduction of the chromosome, the data supports the hypothesis that the hypersensitivity of *recF* mutants may be caused by an inability to resume replication when it is blocked by DNA damage, rather than by a deficiency in recombination. Consistent with this, we went on to show that the recovery of UV-irradiated *E .coli* promoted by the *recF* pathway additionally requires that the replication blocking lesions be repaired, suggesting that the *recF* proteins are primarily needed to maintain the replication fork until repair occurs, rather than to promote recombinational exchanges around the lesion.

Other *recF* pathway genes, *recJ* and *recQ*, were found to participate in the accurate resumption of replication blocked by DNA damage. These gene products were

shown to process the nascent DNA prior to the resumption of DNA synthesis. The processing involves the selective degradation of the nascent DNA on the lagging strand side of the replication fork. It occurs in a manner that may be expected to help maintain the replication fork until replication can resume. Consistent with this, others have shown that these genes affect the sites and frequencies at which illegitimate rearrangements occur following UV-induced DNA damage.

The mechanism by which replication accurately recovers when it is blocked by DNA damage is a critical process which has not been extensively studied in other cell types. However, mutations in human homologues of the RecQ protein have been shown to result in the cancer prone and premature aging disorders of Bloom's syndrome and Werner's syndrome, both of which are associated with increased rates of recombination, suggesting that the mechanism of replication fork maintenance may in many ways be conserved.

During chromosomal replication in *E. coli*, recombination is observed primarily when the normal recovery of replication is inhibited (eg. by unrepaired lesions), suggesting that the recombination and loss of genomic integrity which occurs under these conditions may often result from tolerated, but inappropriate, resolutions to strand pairing events that occur when replication is disrupted.

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One step back, two steps forward.

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Chapter 1

Introduction

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Recombination as a reproductive strategy

Genetic recombination has been observed in essentially all organisms that have been studied and it is clearly an important, sometimes essential, component of many reproductive cycles. However, not all recombination is beneficial for the cell or organism in which it occurs. When it occurs at the wrong time in the cell cycle or the wrong place in the genome, it can create the genomic rearrangements, duplications, or deletions which are almost universally characteristic of cancer cells. It can also lead to the replicational deregulation and overreplication of genomic material, as seen during the lytic replication of many viruses. Thus, in trying to understand the biological role of recombination in a given cellular context, it is important to consider the process, strategy, and products of the reproductive cycle in which it is being studied.

Various reproductive cycles utilize different strategies and yield different products. In the case of eukaryotic organisms, cells may undergo either a sexual reproductive cycle or an asexual one. The sexual reproductive cycle promotes variation, creating progeny that are genetically distinct from the parent cells. That variation is achieved in part because new genetic material is introduced (i.e. through mating)during the reproductive cycle. Additionally however, homologous strand exchanges also appear to be a fundamental mechanism by which variation can be introduced. Following chromosomal replication in meiotic cells, homologous chromosomes are aligned and paired, and numerous strand exchanges are observed. Several techniques, both microscopic and genetic, have documented that recombination occurs with a significant frequency during meiotic divisions (133). In fact, the discovery and concept of genetic recombination has its roots in genetic observations linking traits and their patterns of inheritance following sexual cell cycles.

In contrast, the asexual reproductive cycle is essentially based upon a lack of variation among the progeny. During asexual reproduction, new genetic material is not normally introduced, and the parental genome is duplicated through a processive replication of the template to produce two precise copies that are genetically identical clones of the parental cell. While strand exchanges are fundamental to meiotic cell cycles, they are not generally seen during mitotic cycles (38, 67, 132). Following replication in mitotic cells, the chromosomes are not aligned or paired as is seen to occur during meiosis. Using techniques that label and stain sister chromatids differentially, exchange events between sister chromosomes can be observed with high accuracy (reviewed in (133)). However, very few exchanges are observed in mitotic cells (figure 1A). Furthermore, it has been suggested that the few exchanges which are observed are likely to be caused primarily by the nucleoside analogs (e.g. 5-bromodeoxyuridine or [^{3}H]thymidine)which

are used to label the chromatids for analysis (39, 40, 78, 81). Studies using lower doses of 5-bromodeoxyuridine on *Drosophila* chromosomes revealed no exchanges in a sample of 361 cells (39). A limiting frequency which was calculated as equivalent to less than one exchange event occurring for every twenty replication cycles in human cells (67). By extension to *E.coli*, this frequency would predict that a sister chromatid exchange would occur less than once in more than ten thousand cell divisions.

Consistent with the lack of strand exchange in mitotic cells, those conditions which induce higher frequencies of strand exchanges during mitotic cell cycles often appear to have detrimental effects upon reproductive success (figure 1B & C). In mammalian cells, there is a direct correlation between the frequency with which strand exchanges occur and genomic instability, cell death, and the frequency of carcinogenic transformation (26, 27, 52, 108, 127). In fact, strand exchange frequency is often utilized as a diagnostic marker for both cancer predisposition and the prognosis of cancerous cells to become malignant (14, 15, 25-28, 31, 48, 52, 54, 87, 90, 108, 114, 123, 127). Thus in striking contrast to the situation for sexual cycles, in asexual cycles strand exchanges are not normally observed, nor do they necessarily appear productive when they are observed.

In contrast to asexual cell cycles, recombinational mechanisms do appear to be fundamental to many viral reproductive cycles. Following the replication cycle of Herpes simplex virus type I, a large fraction of the progeny consists of recombinant genomes (4, 46, 106, 128, 136). The recombinational exchanges are intimately linked with the onset of lytic viral replication, and it has been suggested that the strand exchanges are used to initiate multiple rounds of replication simultaneously, allowing the virus to produce much more of its genetic material than it could if it relied upon a unique origin of replication (4, 42, 84). However, rather than discrete chromosomes, this recombinational replication mode results in a large, intricate meshwork of branched genomic concatamers (4, 7, 106, 107, 109, 136). Although not all of the genetic material produced will be packaged or form complete genomes, this strategy is effective for the virus, which relies upon "high volume production" rather than precision for the perpetuation of its genetic message. Because viral replication involves high frequencies of recombination, viruses are often used as model systems to study genetic recombination. Even though the viral recombination occurs within an asexual cell however, the viral replication strategy and products are very different from those of cellular genomic replication. Whereas a single viral reproductive cycle will often package hundreds or thousands of viral genomes from a large mass of genetic material, asexual cellular reproductive cycles replicate each portion of the genome exactly once, creating precisely two complete genomes. It would seem improbable that a replicational strategy like that of the virus would be a productive

mechanism to replicate cellular chromosomes. One interpretation for the viral recombination which occurs could be that it serves as a mechanism by which the virus can escape from the controlled, processive doubling of a genome which occurs in asexual cells. Consistent with this train of thought, many eukaryotic viruses have been found to encode proteins which manipulate or inactivate the cellular proteins, such as p53 and Rb, which suppress inappropriate replication and genomic rearrangements on the chromosome (9, 19, 32, 58, 63, 71, 91, 93, 98, 113, 115, 129, 135). Therefore, while viral systems may be useful in trying to understand the biochemical mechanisms of the recombination process *per se*, it is important to keep in mind that the products and events of this replication cycle may not necessarily reflect the strategy or events which occur during reproduction of the intact cell.

Prokaryotic organisms such as *E. coli* have also been useful models for understanding the mechanisms of genetic recombination. Although bacteria are often thought of as strictly asexual organisms, similar variations in the processes, strategies, and products of different reproductive cycles can also be seen. Like in mitotic cells, the asexual cycle of *E. coli* produces two identical daughter cells through replication of the parental genome. The replication of the bacterial genome is highly processive and does not normally alter the genetic material in the cell. However some other processes in *E. coli* appear to utilize different reproductive strategies. Conjugational reproduction can also occur and several aspects of this process resemble those of a sexual reproductive cycle (reviewed in (53, 68)). During conjugational reproduction, genetic material is transferred from one parent cell to another, recombinant products can be isolated, and as a result of either one or both of these events, the progeny cells contain a different genetic makeup than that of the original parent cell. All of these features are shared with those of sexual cycles.

Recombination is also integral to the reproductive strategy of many bacteriophage. Phage T4 requires recombination to initiate and propagate the massive production of its own genetic material during lytic replication (reviewed in (85)). Phage lambda also exhibits high frequencies of recombination (reviewed in (112)). Also similar to mammalian viruses, many bacteriophage also encode proteins which either inactivate or alter the function of proteins, such as RecD, that appear to suppress inappropriate replication and strand exchanges (79, 86, 96, 121).

Due to the high frequencies of recombination which occur during conjugation and phage replication, these processes are often used as models to characterize the mechanism of recombination in bacteria. However, similar to the case in human cells, the frequency of recombination on the bacterial chromosome is much lower during asexual reproduction,

and several observations would suggest that the recombination which does occur is not productive for replicating or maintaining the genome. While the small size and structure of the bacterial genome prohibits a direct microscopic observation of the number of strand exchanges, density labeling experiments on replicating bacterial chromosomes suggest that the replication is highly processive and does not normally involve detectable levels of strand exchanges (101, 102). Mutations or conditions which increase the frequency of strand exchanges are also associated with genomic instability and poor viability, suggesting that the mechanism of asexual reproduction in bacteria may be analogous to that of human cells in that the recombinational exchanges may not generally be desirable events for the cell during normal genomic replication (94, 102).

Recombination as a mechanism of repair

Although strand exchanges are fundamental to many reproductive cycles, the above observations suggest that they may be neither a predominant nor a productive component of the asexual reproductive cell cycle. In marked contrast to this view however, is the prevailing hypothesis which suggests that recombination is nearly essential to all cellular reproduction, functioning as a major mechanism by which genomic stability is maintained (18, 49, 64, 99, 110). This concept developed from the pioneering study of Clark and Margulies whose goal was to identify genes required for genetic recombination (17). The authors utilized a conjugation assay to screen for mutants which failed to form recombinants during mating. They identified the *recA* gene and showed that although the DNA was transferred during conjugation, no recombinant molecules were produced in the *recA* mutants, suggesting that *recA* was required to catalyze at least one of the steps involved in the formation of recombinants. Additionally, however, the authors made the important observation that *recA* mutants were hypersensitive to UV light during the normal asexual reproductive cycle. To explain this observation, Howard-Flanders in collaboration with Clark suggested the possibility that, since *recA* was required to catalyze one of the steps in recombination, the hypersensitivity to UV light might also be due to a recombinational deficiency- implying that strand exchange may function to maintain the genome in the presence of DNA damage (17, 50).

To test whether RecA can promote recombinational repair of DNA damage, Howard-Flanders et. al. examined the effect of *recA* on the survival of UV irradiated *uvrA* mutants, totally defieceint in the repair of UV photoproducts (51). They found that the survival of a UV-irradiated *uvrA* mutant was higher than that of a *uvrArecA* double mutant (figure 2A). Since *uvrA* mutants are unable to excise UV-induced lesions, they hypothesized that the *recA*-dependent survival could represent a recombinational

mechanism of recovery. Based upon this hypothesis, subsequent studies characterizing *recA* function have focused upon the recovery and survival of *uvr* mutants, with the assumption that the resulting phenotypes must represent a reccombinational mechanism of recovery. These studies demonstrated that in *uvr* mutants, the very limited replication which occurred after UV irradiation resulted in daughter DNA strands that contained gaps and that subsequently underwent high frequencies of strand exchanges (34, 37, 102). Furthermore, in *recA* mutants and certain other *recF* pathway mutants, these daughter strand gaps were shown to persist for an extended period of time. Based upon these experiments, it was proposed that these recombination genes promote strand exchange as a mechanism to reconstruct genomes from the partially replicated sequences of undamaged regions and this led to the general view that recombination is a major repair pathway, required for cellular survival and genomic stability (34, 37, 102) (figure 4B).

An alternative role for recombination proteins

The foregoing proposal relies upon the assumption that since the *recA* gene was discovered in a recombination assay, its primary function must be that of recombination. However, the observation that *recA* function is required during both conjugational recombination and during asexual reproduction could suggest that *recA* has functions unrelated to recombinational processes. Consistent with this, we now know that many of the *recA* dependent effects following UV irradiation are due to its role in the induction of the SOS response which includes the upregulation of the *uvrA* and *uvrB* genes for excision repair and the *umuCD* genes for bypassing replication blocking DNA lesions ((23,33) and discussed below). Since high frequencies of recombination during asexual reproduction appear to be detrimental to cellular reproduction, it would be useful to consider that RecA function may not necessarily promote recombination during asexual reproduction.

From this point of view, it is then worth reexamining whether a recombinational deficiency is able to account for the UV-hypersensitivity of *recA* mutants. Although the original recombinational studies demonstrated that strand exchanges *can* be induced at unrepaired lesions, they could also be interpreted to suggest that the recombination does not significantly contribute to the cellular recovery - a conclusion which one might draw considering the dramatically higher survival of wild type cells (figure 2B). The conclusions of Howard-Flanders et. al. (1969) focused upon repair deficient mutants of *E. coli* which were dependent upon *recA*-mediated pathways for survival. These excision repair deficient mutants survived UV doses which produced up to 50 lesions scattered throughout their genome. However, the wild type *E.coli* used for comparison in these studies were found to recover from irradiations producing in excess of 2500 lesions

without suffering any significant loss in survival (figure 2C). Since the *E. coli* which were dependent solely upon excision repair were unable to survive more than 20 lesions, a strictly recombinational function for RecA in the recovery process fails to explain the recovery of wild type cells. From this perspective, the above results suggest that the ability of RecA to promote recombination is almost insignificant for cellular viability. Nevertheless, cell survival in the presence of DNA damage absolutely requires some function of *recA*. Thus, rather than an independent recombinational pathway, the survival curves imply that the vast majority of events catalyzed by RecA are also dependent upon proficient excision repair. Because the authors were originally testing only for the presence of recombination, they concluded that RecA *could* function independently. However, based upon the survival of wild type cells, their results suggest that the operation of RecA is almost entirely epistatic with excision repair in the recovery process.

Extending the idea that recombination could function as an independent recovery pathway, other studies in *uvr* mutants have demonstrated that following low doses of UV, DNA replication partially recovers and that strand exchanges are induced at the unrepaired lesions (34, 37, 102). Since RecA was thought to be a recombination-specific protein, the common interpretation of these results has been that the strand exchanges represent a mechanism by which cells are able to reconstruct intact genomes through recombinational exchanges. However, if *recA* function is not limited to its role in recombinational processes, we might conclude that the exchanges are just as likely to be deleteriously scrambling the genome as they are to be productively reconstructing it. From this perspective, the recombinational exchanges are primarily observed to occur in mutants which are unable to repair DNA lesions. Additionally, despite the relatively low doses used in recombinational repair studies, these strand exchanges are only detected when there is a significant inhibition of replication, loss of viability, and inhibition of cell division. In wild type cells, although these strand exchanges are primarily suppressed, the recovery is much more efficient with no loss in viability even at much higher doses of irradiation (51, 72, 130). The fact that significant levels of strand exchanges are only observed in populations with high levels of cell death and inhibited cell division could suggest that those cells which are undergoing strand exchanges are not productively reconstructing genomes. Rather, the strand exchanges could be interpreted as a failure to maintain semiconservative replication when the lesion is not (or can not be)repaired normally. Although strand exchanges may not be required for recovery and survival in these cells, some other function of *recA* is still required, suggesting that the mechanism of recovery in repair proficient cells may be very different from that in repair deficient cells.

If strand exchange is not productive, then why is *recA* function required in the presence of DNA damage? Several studies suggest that the defect in *recA* mutants has its impact primarily when DNA damage is encountered by the replication machinery. If *recA* mutants are pretreated such that ongoing replication is either inhibited or allowed to complete the replication cycle, they are found to be much more resistant to UV irradiation (44, 47, 51, 118, 119). This is in contrast to *uvr* mutants whose sensitivity to UV irradiation is not altered by these types of treatments, suggesting that the hypersensitivity of *recA* mutants may relate to a problem for replication caused by the DNA lesions rather than the lesions themselves (22, 44, 118). Additional studies have also shown that in *recA* mutants, replication is severely inhibited following UV-irradiation and fails to recover (50, 51, 59). At high UV doses, the arrest of replication occurs almost instantaneously, while at lower doses, where there is a large average distance between lesions, replication is seen to continue for a short period before the inhibition occurs, consistent with replication arrest occurring at the DNA lesion itself (47, 50, 122).

Additional clues as to RecA's function at the replication fork were provided by the early studies of Horii and Suzuki (47). By examining the fate of DNA made prior to irradiation, they found that although wild type cells were able to protect their genomes, a rapid and complete degradation of the entire genome occurred in the *recA* mutants. They found that the genomic degradation only occurred if the *recA* mutants were actively replicating at the time of exposure (figure 3). Through pulse-chase labeling of the DNA, they were also able to show that the genomic degradation initiated from the blocked replication forks and then processively degraded back from these points. At lower doses of UV, when there was a large distance before a replication fork encountered a lesion, the authors found that the DNA synthesis continued for a short period before the degradation began. Once it began however, the degradation occurred with the same kinetics as at the higher doses. These observations led the authors to propose that RecA function was required to protect and maintain the strands of the replication fork when it becomes blocked by DNA damage (47). This was a most important observation and conclusion.

The proposal of Horii and Suzuki (1968) for RecA function during replication is interesting because it would explain why RecA is required in the presence of DNA damage even if strand exchange does not occur. Importantly however, their proposal is also be consistent with the known biochemical properties of the RecA protein. The same enzymatic activity could explain how RecA could be required for both recombinational processes and replicational processes. Biochemical studies have shown that purified RecA will progressively bind and pair single stranded DNA with homologous duplex DNA in a 5'-3' direction. The product of this reaction creates a RecA protein filament which is

bound to a triple stranded DNA structure (for reviews see (64, 99, 110)). During processes which involve recombination, this activity is thought to be critical for bringing together homologous strands from different DNA molecules. However during asexual reproduction, there is no need or desireability for recombinational exchange since, as emphasized earlier, a single genomic template is duplicated through processive replication. Although strand exchanges are not normally observed, RecA function is still required for the resumption of processive replication when it has been blocked by DNA damage. One attractive possibility for how this same biochemical activity of RecA may be required for both recombinational processes and a nonrecombinational role at the replication fork is suggested by the structure created during semiconservative replication. During semiconservative replication, both strands of the DNA template are replicated concurrently in a 5'-3' direction. While the leading strand can be replicated continuously without interuption, replication of the lagging strand template must occur discontinuously, periodically reinitiating synthesis as the replication machinery moves processively down the template. This coordinated replication of both strands implies that at any given time the region immediately behind the replication machinery will contain a single stranded region on the lagging strand template in addition to the newly replicated leading strand. In the event that the replication of the template becomes blocked, this region behind the replication fork is essentially identical to the substrate upon which RecA acts during recombinational processes. However unlike recombinational processes, pairing between the replicated leading strand duplex and the nonreplicated lagging strand template would stabilize the DNA strands at the replication fork, rather than bringing together strands from two different DNA molecules as occurs during recombination. The rapid degradation of the genomic DNA which occurs when replication encounters a DNA lesion in *recA* mutants provides experimental support for the view that this pairing may be required to protect the replication fork from degradation. As mentioned earlier, the dependence of *recA* function upon excision repair for efficient recovery may indicate that such pairing is maintained until after the lesion has been repaired. Maintaining these strands until repair occurs, rather than exchanging them, would preserve the accurate and semiconservative duplication of the template. If the lesion can be repaired, there is no need for strand exchange. Thus, although recombinational strand exchanges may occur in repair deficient mutants when the lesions cannot be repaired, the dependence of *recA* function upon the *uvr* genes implies that the recovery process in wild type cells may require that *recA* maintains the replication fork until the arresting lesion has been removed (figure 4A).

This type of model may also better explain the inhibition of replication which is initially observed to occur after UV irradiation. Classical recombination repair models

predict that replication should resume downstream from the blocking DNA lesion. Yet, studies have shown that replication is severely inhibited in both excision deficient mutants and *recA* mutants. The inhibition in *recA* mutants occurs despite the fact that the DNA lesions are removed from the template at rates comparable with those in wild type cells (35). Thus, even if the downstream reinitiation were relatively inefficient, one would predict that the initial recovery of *recA* mutants should be similar to wild type cells since after encountering an initial lesion, the downstream template should be restored, thereby allowing replication to resume normally while leaving a single gap behind at the site of disruption. The lack of recovery in *recA* mutants is perhaps more consistent with the idea that replication remains processive, but delayed until the blocking lesion is either repaired or that replication is able to resume by incorporation of a nucleotide through a lesion bypass mechanism (see below).

In *uvr* mutants, there is also a severe inhibition of replication after UV-irradiation (101, 105). However, the limited replication which does occur after irradiation in either *uvr*$^-$ or *rec*$^-$ *E.coli* results in daughter strand gaps (36, 100-102). These observations have classically been interpreted in support of a recombinational repair model in which replication reinitiates downstream from the DNA lesion and then, at a later time, the intact genomic molecules may be reconstructed from the partially replicated templates. The consequences of the general inhibition of replication and DNA degradation caused by DNA damage should also be considered with respect to the fragmented DNA that have been observed. It seems possible that the process of degradation itself may create a significant increase in 3' termini that could potentially be extended by polymerases. Radioactive label incorporation in the experiments could then give an impression of fragmentary replication even though the genomic replication has been inhibited. By analogy to human cells, it is useful to consider the xeroderma pigmentosum variant (XPV)complementation group. The phenotypes exhibited by these cells after UV-irradiation bear a striking resemblance to those of *recA* and *recF* mutants. Like *recA* and *recF* mutants, irradiated XPV cells have been found to be hypersensitive to UV irradiation, even through the UV-lesions are removed from the genome at rates similar to those in normal cells; and the DNA synthesized by XPV cells following irradiation has been shown to be fragmentary and not joined into larger fragments (69). Also similar to *recA* or *recF* cells, it has been shown that the replication in XPV cells does not exhibit the high frequencies of strand exchanges which are observed in classical, excision deficient, XP complementation groups (24, 77, 132). These early observations led to the interpretation that XPV cells were defective in a post-replication recombinational repair process very similar to that postulated for E. coli (1, 24, 43, 69). However, more recent studies have

demonstrated that replication is blocked at a DNA lesion on a plasmid template in extracts from XPV cells but not in extracts from normal cells (21, 116). Recently, the XPV gene product was cloned and shown to function as a DNA polymerase which allows the incorporation of nucleotides opposite the DNA lesion in the template strand and then resume (55, 80). Thus in the case of XPV cells, the cellular phenotypes of a recombinational repair defect (i.e. fragmentary replication and a failure to join fragmented products)are produced instead by a defect which fails to allow replication to bypass and resume beyond replication blocking lesions. Given the similar inhibition of replication and the DNA degradation which are seen in *recA* and *recF* mutants, it seems reasonable to suspect that these phenotypes could also be produced by a failure of replication to overcome blocking DNA lesions.

General consequences and implications of recombination during replication

It is important to make the distinction that we have been discussing types of DNA damage (i.e. UV photoproducts) that do not directly cause strand breaks, particularly double strand breaks. In the unique and relatively rare situations in which cells must deal with double strand breaks (or with interstrand crosslinks) in which both strands of the DNA are damaged within the same short fragment, it is likely that there is an essential role for the process of recombination to effect repair. At the same time, it is important to keep in mind that asexual reproduction produces two complete genomes with an identical linear order. As such, cellular processes should generally promote this end product. Yet several aspects of recombination could potentially compromise this end. Previous recombinational models have sometimes suggested that to facilitate the repair of a lesion-containing DNA strand, the undamaged sister chromosome is incised and used as a donor. Although this mechanism is possible to perform with pencil and paper (with some difficulty), it is a complex task to request of "blind" enzymes, which must not only initiate these events but also contain them and prevent them from occurring when they are not supposed to. The repair process itself has the potential to make a bad situation worse by incising the cell's only intact copy of a genomic region and would require several, yet unknown enzymatic steps capable of "sensing" which, when, and where undamaged sequences should be incised and then re-replicated.

An additional, yet often neglected consideration of asexual reproduction is how cells are able to control and limit replication to an exact doubling of the genome. Even in the relatively simple genome of E. coli, how this is accomplished is more than a daunting task to imagine from a purely enzymatic approach for a tightly packaged string of over four million base pairs. Yet, this task becomes even more complicated in the event that

processive replication is allowed to initiate from a recombinational intermediate. Following recombinational initiations of replication, the genomic replication will not proceed through a point in which each sequence has been duplicated exactly once. Instead, these initiations would be expected to create portions of the chromosome which have been over replicated while leaving other regions unreplicated (65, 66). The resolution and recovery of intact genomes once this has occurred would require some form of "enzymatic memory" to reconstruct the original linear order of a genome which no longer exists in the cell. The requirement for such enzymatic memory could clearly be avoided if the cell is able to maintain processive replication. If genomic replication remains semiconservative, then following the initiation of replication on the chromosome, whether it initiates from one origin or many origins, a point will be reached at which the entire sequence has been duplicated exactly once. Thus, although recombinational replication appears to be productive in the reproductive strategy of a phage or virus, it might be expected to have a deleterious effect on the survival and genomic stability of the cell as discussed earlier.

Experiments characterizing a phenomenon in *E. coli* called inducible stable DNA replication (iSDR) reflect the deleterious effects of recombinational initiations during genomic replication. Chromosomal replication in *E.coli* is initiated through a tightly regulated process which involves the synthesis of new proteins each time chromosomal replication is initiated (45, 74). Through tight regulation of the time and place a replication fork is initiated, the cell ensures that upon division, each daughter cell receives an equal and precise copy of the genetic material. However, in the presence of protein synthesis inhibitors, new rounds of replication can not be initiated from *oriC* and replication ceases after the ongoing rounds of replication are completed (45, 74). When the inhibitors are removed, replication eventually reinitiates without significant loss of survival as the proper symmetry of replication forks per chromosome has been maintained throughout the treatment. However, if the cells are first induced for the SOS response by treatments such as UV irradiation or thymine starvation, then replication is seen to continue for a much longer period- independent of new protein synthesis (60-62). Several lines of evidence have demonstrated that the replication occurring under these conditions initiates from recombinational substrates created by the DNA damage, allowing the cell to produce several times the amount of DNA beyond which genomic replication would normally have completed (reviewed in (61)). However, the recombination which occurs under these conditions has a drastic effect upon cell survival. Cells which have been starved for thymine rapidly lose viability (20, 74). Nakayama has correlated the loss of viability with strand exchanges occurring throughout the genome (88). That it is the recombination occurring at the DNA damage, rather than the DNA damage itself, that causes cell death is

suggested by analysis of mutants in the *recF* pathway which are more resistant to thymine starvation (88, 89). Mutations in the *recF* gene of E.coli confer a partial resistance to thymine starvation. Yet, *recF* mutants are hypersensitive to DNA damage. If DNA damage created by the thymine starvation were responsible for the death occurring in these cells, one might expect that *recF* mutants would be hypersensitive to thymine starvation. Instead however, Nakayama correlated the survival of thymine starved *recF* mutants with a decreased ability to form recombinagenic structures during starvation (88). Kogoma has shown that these mutants are less able to initiate recombinagenic replication (3, 61). These observations would be consistent with the idea that the replication initiated from recombinational substrates may be detrimental to cell survival.

Genomic replication as a single process with multiple components

Although both models deal with the resumption of replication, recombinational models generally suggest that RecA function promotes the initiation of replication from new sites. By contrast, the model discussed here of maintaining replication fork stability implies that during asexual reproduction, *recA* protein plays a fundamental role in *preventing* the initiation of replication from new sites. These contrasting possibilities are interesting to consider with respect to other cellular processes which involve both RecA and DNA replication.

Consider the generalization that recombination in *E. coli* appears to require activities which promote the formation of an extended single strand 3' end- presumably to utilize as a substrate for replication initiation (60, 64, 110). Although *recD* encodes an exonuclease which has been primarily characterized as a recombination enzyme, RecD activity is found to preferentially degrade the 3' recombinagenic ends and is needed to suppress the frequency of recombination (11, 29, 30, 121). Further, if RecD is inactivated, replication of phage or plasmid molecules is no longer limited to an exact doubling but enters a runaway replication pattern similar to that in lytic phage replication (2, 92, 96). Similar effects may also occur on the *E. coli* chromosome but have not been directly examined (2). Thus, in many ways, RecD could be thought to prevent the recombinagenic initiations of replication.

Other recombination genes, such as *sbcA* and *sbcB*, were isolated because these genes, when mutated, were found to restore recombination to *recBC* mutants in recombination assays (120). However, the *sbcA* mutation upregulates the expression of a normally suppressed double stranded 5'-3' exonuclease, exoVIII, of a cryptic prophage (41, 56). Thus in wild type cells, this activity which creates recombinagenic substrates is normally turned off. Similarly, *sbcB* mutations inactivate ExoI, an exonuclease which

normally degrades 3' DNA ends (95). Furthermore, ExoI has been shown to associate with both RecA and SSB (5, 6, 70, 83). Thus the properties of ExoI suggest that *in vivo*, any recombinagenic 3' DNA ends bound by RecA might be expected to be degraded by ExoI. The cellular function of these enzymatic activities is difficult to rationalize if we assume that they promote recombination. However, in the context that it is desirable for the cell to maintain semiconservative replication during asexual reproduction, these activities make much more sense. All of these activities, as expressed in the cell, appear to suppress or eliminate the formation of potentially recombinagenic 3' DNA ends from the cell. If replication only extends the 3' ends of the replication fork, genomic stability will be maintained.

In addition to *recA* , other processes also function coordinately with the replication fork and bear consideration with respect to how they may interact. The mismatch repair system is thought to function by replacing misincorporated bases on the newly synthesized strand of the DNA template (33, 82). This process requires that one newly synthesized strand be paired with a parental template strand such that the incorrect base in the newly synthesized strand can be recognized. In contrast, a strand exchange process at the replication fork creates newly synthesized duplex and parental duplex, i.e. portions of DNA which are paired conservatively rather than semiconservatively. Thus one might predict that the presence of the mismatch system would inhibit strand exchanges from occurring. Perhaps reflective of this cellular arrangement, the mismatch repair proteins of *E. coli* have been shown to inhibit *recA*-mediated strand exchanges *in vitro* and inhibit chromosomal recombination *in vivo* (82, 94, 134). A similar inhibitory effect upon recombinational events has also been observed by the mismatch repair pathways in both yeast and humans (10, 12, 13, 16, 104).

When replication becomes blocked by DNA damage in *E. coli*, the RecA protein itself is central to the regulation and induction of over twenty genes, a cellular response which has been termed the SOS response (reviewed in (33)). In addition to their regulatory requirement for *recA*, many of the induced genes which deal with the recovery of replication involve a functional interaction with RecA as well. The *umuCD* and *dinP* genes which are under SOS control are thought to encode proteins which help replication machinery proceed to replicate through DNA lesions that otherwise block replication. At least for UmuCD function, lesion bypass is also known to require RecA (57, 73). If RecA function simply maintains the blocked replication fork, lesion bypass would appear to be a potentially useful event if the lesion is not or cannot be repaired. However, if RecA function were to promote strand exchange at these sites, it would appear to function in opposition to the bypass proteins. Consistent with the idea that the resumption of

replication must either involve repair or translesion synthesis, Evelyn Witkin found that a *recA* mutant which remained proficient in recombination failed to recover replication in the absence of umuC (131). Furthermore, as was observed with the mismatch proteins, the gene products involved with lesion bypass all appear to inhibit RecA-mediated strand exchange *in vitro* and to suppress recombination *in vivo* (8, 97, 111, 117, 126). Other proteins have also been found to inhibit recA-mediated strand exchange. Although discovered as a recombination protein, initial studies on purified RecF showed that rather than promote strand exchange, it had an inhibitory effect when in the presence of RecA (75). Since RecF was considered to function as a recombination protein, however, this observation was dismissed and subsequent experiments have focused upon finding conditions in which this inhibitory effect does not occur (76, 124, 125).

The excision repair genes, *uvrA* and *uvrB*, are also induced during the SOS response . Recently, this induction of these genes has been shown to be required for the efficient removal of the DNA lesions from the genomic template at higher doses of UV irradiation (23). It was argued that the induced levels are likely to be needed for efficient survival at higher doses so that the replication blocking lesions can be removed.

Taken together, these observations suggest that although RecA may promote strand exchange during recombinational processes, during chromosomal replication, in the presence of the replication machinery and its associated proteins, the enzymatic activity may help maintain the processive, semiconservative replication of the genome. The experiments presented in the following chapters will deal primarily with the requirement for *rec* genes in the presence of UV-induced DNA lesions. It was my hope and intention to offer this as a novel (and useful)perspective by considering the functions "*rec*" genes may have in the cell, other than those required for recombination.

I pursue this perspective in the following chapter by focusing upon the *recF* and *recR* genes of *E.coli*. Similar to the *recA* gene, these genes were isolated as recombination genes which were required for congujational recombination when the *recBCD* recombination pathway has been inactivated, and studies have classically focused upon their recombinational functions. However, the observation that, in an otherwise wild type background these genes are fully proficient in conjugational recombination assays although they are hypersensitive to UV irradiation, suggested that recombinational functions alone for *recF* are unable to account for the UV hypersensitivity of these mutants. With this perspective in mind, we examined the effect the *recF* mutation had on replication following UV irradiation and found that the recovery of replication required both the *recF* and *recR* genes. In their absence, replication was severely inhibited by UV irradiation, failed to recover, and resulted in degradation of one half of the nascent DNA at

the growing fork. These results are the key observations which led us to consider the similarities between the substrates which are required for recombination to occur and those which exist at the disrupted replication fork.

In Chapter 3, we compare the hypothesis that the UV hypersensitivity of *recF* mutants could be explained by a failure to resume replication from disrupted replication forks to that of previous models which suggest that the hypersesitivity of *recF* may be due to an inability to repair daughter strand gaps through a recombinational process. Since the recombinational models suggest that RecF function should be independant of excision repair, we examined the effect that each mutation has on the survival and recovery of replication following UV irradiation. The results suggest that the recovery requires both *recF* and excision repair functions, suggesting that the ability to enhance survival by recombining around blocking DNA lesions is limited.

The final chapter further characterizes the mechanism by which replication recovers following UV irradiation by focusing upon the degradation of the nascent DNA which occurs prior to the resumption of DNA synthesis. We found that the degradation required the *recJ* and *recQ* gene products. These genes have both been classified in the *recF* pathway of genes based upon their recombinational phenotypes. *In vivo*, we found that these genes control the preferential degradation of the lagging strand of the nascent DNA at blocked replication forks prior to resumption, an activity that is consistent with the known polarities of the RecQ helicase and the RecJ nuclease, respectively. These results were interesting because homologs of the *recQ* gene in other organisms have been found to play critical roles in maintaining semiconservative replication and suppressing strand exchanges. In *E. coli*, the *recQ* gene activity appears to increase the single stranded region at blocked replication fork. This action would be predicted to create a much larger substrate upon which the RecA protein may bind and stabilize and we discuss these results in relation to both the recombinagenic and replicational properties of *recJ* and *recQ*.

Figure 1. Strand exchange in human cells. Mitotic chromosomes which have been labeled and stained such that sister chromatid exchanges can be observed. Chromosomes from A.)normal human lymphocytes, B.)a patient with Bloom's syndrome, a cancer prone genetic disorder, and C.)following treatment with MMS, a DNA damaging agent known to induce sister chromatid exchanges. (Photos taken from (103, 132) with permission)

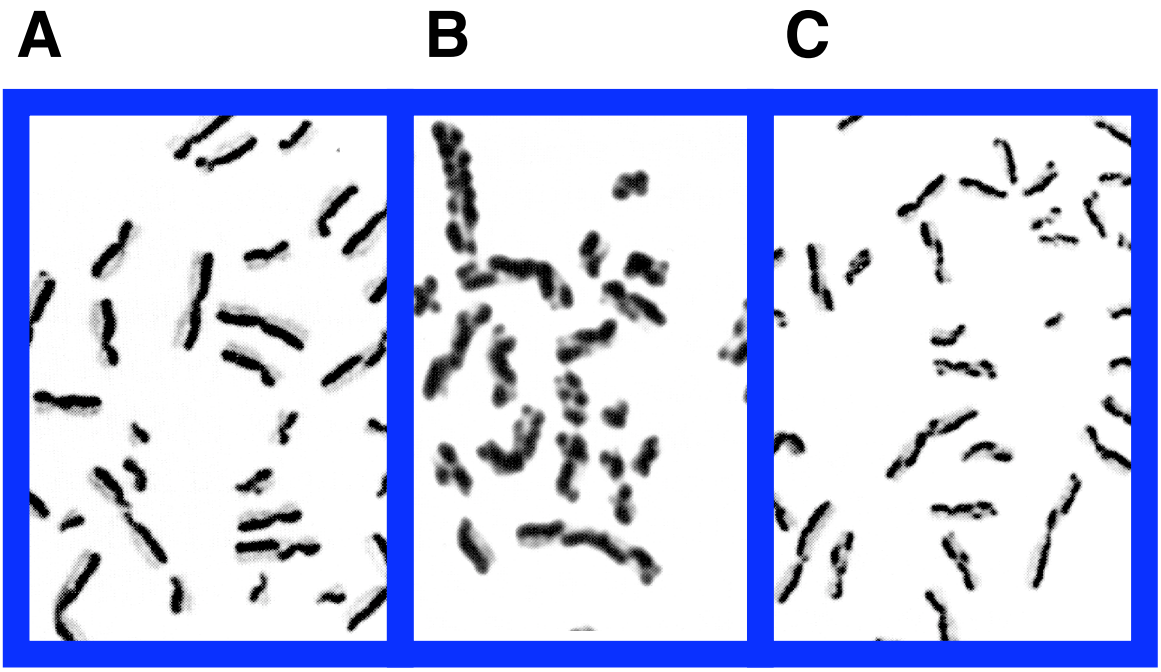


Figure 2. The survival of various *E. coli* mutants following UV irradiation. A.) Focusing upon the question of "Can recombination promote recovery following UV irradiation?" it has been suggested that since a uvrA mutant is more resistant to UV-irradiation than a uvrArecA mutant, recA may promote survival by recombining around DNA lesions. B.) However, focusing upon the question of "How do cells recover following UV irradiation?" it is possible to conclude that recombination promoted by recA may not significantly contribute to the recovery which is seen in wild type cells. C.) Based upon these survival curves, the average lethal number of lesions per chromosome is plotted for each strain. (Adapted from (51))

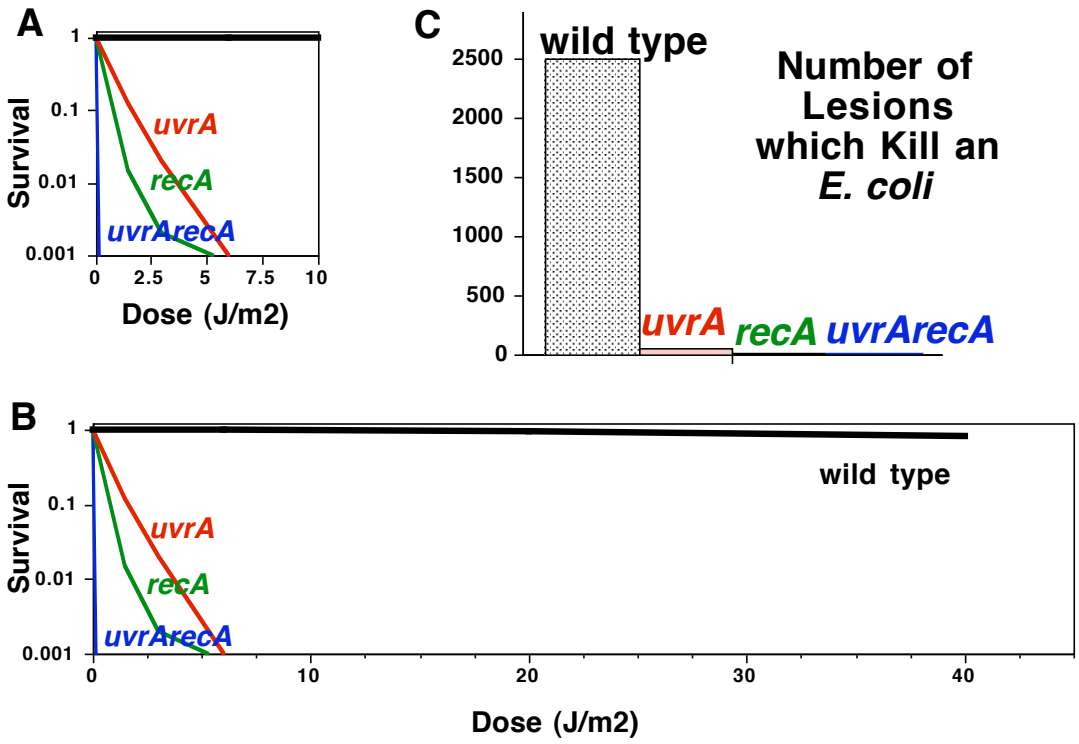


Figure 3. Genomic degradation in UV-irradiated recA mutants. The degradation of DNA made prior to UV irradiation in a recA mutant as followed by acid precipitable counts remaining. Replicating or nonreplicating recA mutants were prelabeled with ^{3}H thymine, placed into nonradioactive medium and irradiated with $5J/m^2$. (Adapted from (47))

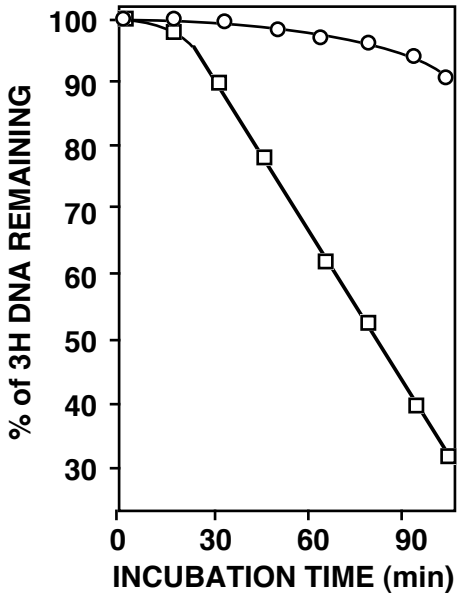
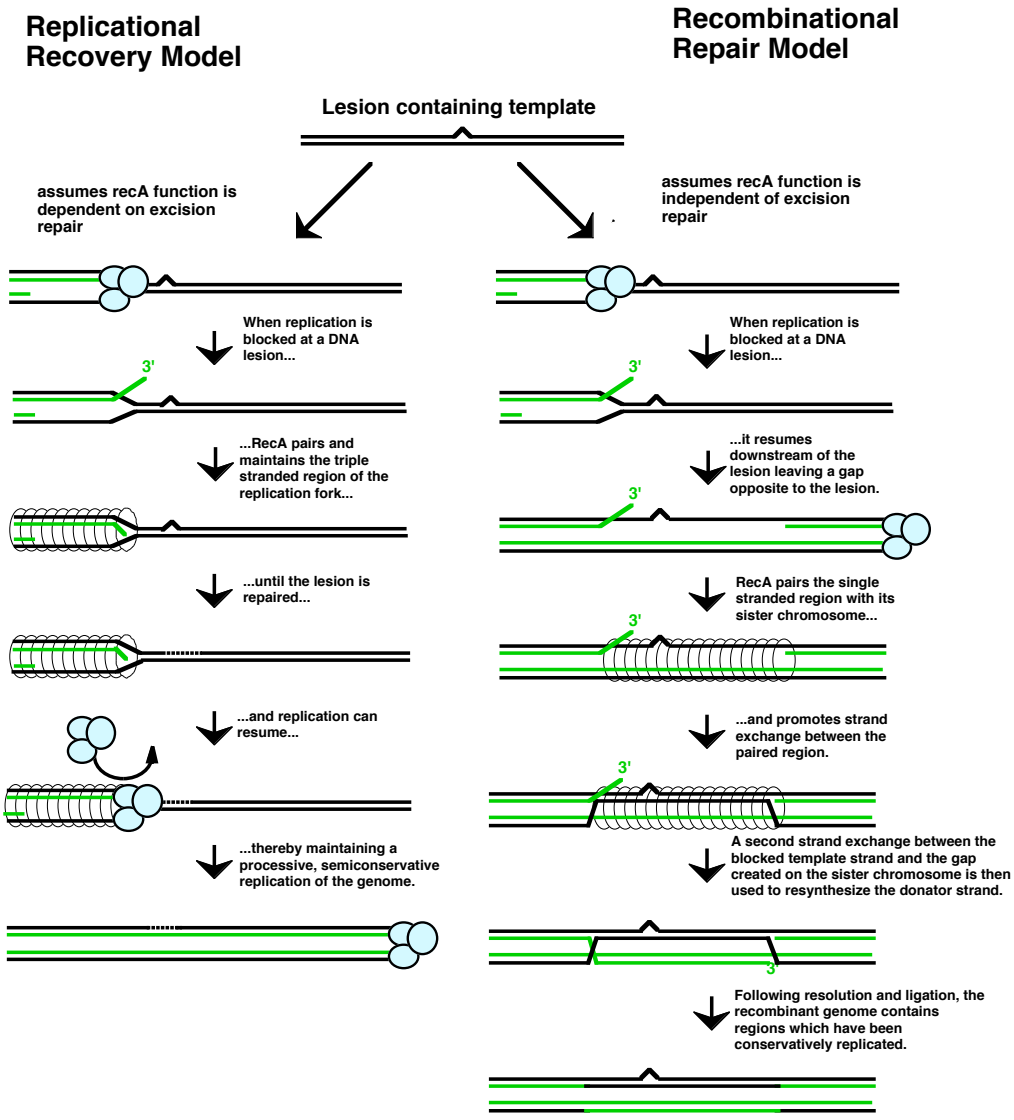


Figure 4. Models for *recA* function during replication of damaged DNA. A.)Replicational recovery model B.)Recombinational repair model.



Observations in wild type cells	Observations in excision repair mutants
• Good Survival	• Extremely Poor Survival
• Efficeint recovery of replication	• Inhibited recovery of replication
• Semiconservative products, without strand exchanges	• Recombinant products, with high frequencies of strand exchanges

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Chapter 2

recF and *recR* Are Required for the Resumption of Replication at DNA Replication Forks in *Escherichia coli*.

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E. coli containing a mutation in *recF* are hypersensitive to UV. However, they exhibit normal levels of conjugational or transductional recombination unless the major pathway (*recBC*) is defective. This implies that the UV sensitivity of *recF* mutants is not due to a defect in recombination such as occurs during conjugation or transduction. Here, we show that when replication is disrupted, at least two genes in the *recF* pathway, *recF* and *recR*, are required for the resumption of replication at DNA replication forks, and that in their absence, localized degradation occurs at the replication forks. Our observations support a model in which RecF and RecR are required to reassemble a replication holoenzyme at the site of a DNA replication fork. These results when taken together with previous literature, suggest that the UV hypersensitivity of *recF* cells is due to an inability to resume replication at disrupted replication forks rather than to a defect in recombination. Current biochemical and genetic data on the conditions under which *recF* mediated recombination occurs suggest that the recombinational intermediate may also mimic the structure of a disrupted replication fork.

Introduction

In E. coli, recombination is classically thought to occur through one of two pathways termed the *recBC* (major) pathway and the *recF* (minor) pathway (24, 54). *RecBC* mutants were originally identified because of the 10^2- to 10^3-fold reduction in their recombination efficiency (10). Further characterization showed that *recBC* mutants were also hypersensitive to UV and X-rays and had a reduced plating efficiency (4, 10). Biochemical studies have since shown that RecBC forms a DNA helicase which associates with a nuclease, RecD, and is thought to unwind and process the DNA ends required for the initiation of strand invasion during recombination (24, 54).

The *recF* pathway of recombination is less well understood. *recF* was identified by screening mutagenized *recBC* cells to isolate the genes required for the 0.1 to 1 percent of recombination remaining in these cells (16). However, when the *recF* mutations were moved into a *recBC*$^+$ background, the recipient cells were found to have normal levels of recombination. Although not deficient in recombination, these cells were as sensitive to UV as were *recBC* cells and they also displayed a low plating efficiency (46). Purified RecF protein has been shown to preferentially bind gapped DNA in the presence of ATP but its function in vivo has not been determined (15, 33, 57). The RecR protein is also in the *recF* pathway and has been shown to interact with the RecO protein (57). Both RecO

and RecR are epistatic with RecF and are thought to function at a common, yet unknown, step in the recombination process (24, 57).

Several studies have shown a link between DNA replication and the *recF* pathway of recombination. Early studies of phage lambda showed that recombination dependent on either the *recF* pathway or a *recF* homologous pathway required the presence of replication (55). Certain forms of aberrant replication such as plasmid linear multimer formation and rifampicin resistant plasmid replication, also have been shown to be dependent upon *recF* (25, 35). Genes belonging to the *recF* pathway are also required for cells to undergo "thymineless death", a process by which cells rapidly die in the absence of thymine (6, 32, 39). Here as well, aberrant replication has been shown to be occurring during the process (21, 34).

Another phenomenon linking the *recF* pathway to replication is long patch excision repair (8, 9). Cooper and P.C.H. found that the size distribution of repair patches in UV-irradiated E. coli was bimodal (9). Short patches appeared at early times and were shown to be due to normal nucleotide excision repair. *recF* -dependant long patches were observed at the time the cells recovered DNA replication, and these were primarily localized at DNA replication forks (7). The long patches were found to be either 1500 bp or greater than 9000 bp in size, corresponding to those expected for Okasaki fragments on the lagging strand and leading strand DNA synthesis, respectively.

At the level of genomic organization, both *recF* and *recR* also appear to be linked with replication. The *recF* gene is found in the same operon as the *dnaN* gene (the beta subunit of the replication holoenzyme), while the *recR* gene is found in the same operon as the *dnaXZ* gene (the tau and gamma subunits of the holoenzyme) (11, 40, 43).

While *recF* and *recR* mutants have relatively subtle phenotypes with respect to recombination, their UV sensitivities are comparatively dramatic. We have considered the possibility that recombination is not the primary function of *recF*. By studying why *recF* causes hypersensitivity to UV, we hoped to gain a better understanding of its function *in vivo*. We have found that the resumption of DNA replication from existing replication forks requires both the *recF* and *recR* genes.

Materials and Methods

Bacterial Strains. SR108 is a *thyA36 deoC2* derivative of W3110 (36). HL919 (SR108*recF349 tnaA300::*Tn*10*) and HL920 (SR108*recR252:*Tn*10-9*) were made by P1 transduction of the *recF349 tnaA300::*Tn*10* and *recR252::*Tn*10-9* markers from strains JC15359 and AM207, respectively (47). HL921 (SR108Δ(*srlR-recA*)*306::*Tn*10*) was made by P1 transduction of Δ(*srlR-recA*)*306::*Tn*10* from JC10289 (58). HL922 (SR108*recB21C22 argA81::*Tn*10*) and HL923 (SR108*recD1011 argA81::*Tn*10*) were

made by P1 transduction of *recB21C22 argA81::tn10* from strain V1307 and *recD1011 argA81::*Tn*10* from strain V220 (1, 5). The *recF*, *recR*, *recA*, and *recBC* phenotypes were checked by UV sensitivity. The *recBC*, and *recD* phenotypes were checked by their ability to support growth of phage T4 gene2$^-$ mutants (49). SR1601 and CAG12156 carry a *uvrA::*Tn*10* marker and *uvrC::*Tn*10* marker respectively (3, 50).

Survival studies. UV irradiations used a 15 watt germicidal lamp (254nm, 0.6 J/m2/sec at the sample position). Cells were grown in Davis medium supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μ g/ml thymine (DGCthy media). Inoculated cultures were incubated for 2 to 3 days prior to irradiation to obtain stationary phase cells. Log phase cultures were typically inoculated from stationary phase cultures and grown to an OD_{600} between 0.2 and 0.3 (approximately $5*10^8$ cells/ml). Chloramphenicol pretreatment (150 μ g/ml) was for 3 hours before the cultures were filtered on Millipore 0.45 μm HA filters and resuspended in fresh medium. All experiments were carried out at 37˚C.

Time course of replication recovery. Cells were grown in DGCthy media containing 0.5μCi/ml [^{3}H]thymine to an OD_{600} of 0.2 at which point half the culture received an incident dose of 25 J/m^2. The incorporation of ^{3}H into the DNA was measured by averaging duplicate, 0.2 ml samples precipitated in 5% cold trichloroacetic acid filtered onto Whatman glass fiber filters.

Density labeling of replicated DNA. Cells were grown in DGCthy media containing 0.2μCi/ml [^{14}C]thymine to an OD_{600} between 0.2 and 0.3 before being harvested by filtration and resuspended in DGC media containing 10μg/ml 5-bromodeoxyuridine. Half the culture received 25 J/m2, each half received 0.5μCi/ml [^{3}H]thymine, and was then incubated for 1 hour. Cells were pelleted and lysed in 0.4ml NET (100mM NaCl, 10mM Tris pH8.0, 10mM EDTA) containing 1mg/ml lysozyme and 100 μg/ml RNaseA at 37C for 30 minutes. Ten microliters of 10mg/ml proteinase K and 10μl of 10% sarcosyl was added, and incubation continued for 1 hour at 65C. The solution was then subjected to isopycnic alkaline CsCl gradient sedimentation as described previously (53). Thirty fractions were collected from each gradient on Whatman#17 paper. ^{14}C and ^{3}H was determined by scintillation counting.

DNA degradation following UV irradiation. Cells were grown in DGCthy media containing 0.2μCi/ml [^{14}C]thymine to an OD_{600} between 0.2 and 0.3. Thirty seconds before harvesting by filtration, 1μCi/ml [^{3}H]thymidine was added to the culture. Cells were washed with 1X Davis medium, resuspended in nonradioactive DGCthy media, and given a dose of 25 J/m^2. ^{14}C and ^{3}H remaining in the DNA was measured as before (see time course of replication).

Completion of ongoing rounds of DNA replication and FACS analysis. Cells were grown in DGCthy media containing 0.5μCi/ml [^{3}H]thymine to an OD_{600} of 0.2 at which point chloramphenicol or rifampicin was added (150 μg/ml). ^{3}H incorporation into the DNA was measured as before (see time course of replication). For FACS analysis, the same procedure was used except 10μg/ml cephalexin (inhibitor of septation) was also added (2). After 3 hours, cells were fixed in 70% ethanol. Staining was done in 50mM Tris ph7.5, 1.5mM $MgCl_2$, 100mM NaCl, and 20μg/ml chromomycin A3. FACS analysis was performed using a EPIC753 flow cytometer (Coulter Inc. Haileigh FL) at 457nm excitation. Elite software (Coulter Inc.) was used for analysis (2).

Results

The UV sensitivity of *recF* and *recR* mutants correlates with DNA replication. We found that the UV sensitivity of *recF* and *recR* cells was dependent upon the replication state of the cells. When wild type, *recF*, and *recR* cells were grown to stationary phase prior to irradiation they were more resistant to UV than were exponentially growing cultures (Fig.1a). Similarly, when the cells were pretreated for three hours with chloramphenicol, a protein synthesis inhibitor, they were also more resistant to UV irradiation (Fig.1b). Inhibition of protein synthesis has been shown to prevent the initiation of new rounds of DNA replication (14, 32). Treatment with chloramphenicol for three hours allows the ongoing rounds of replication to be completed (13, 26). By contrast, the UV hypersensitivity of excision repair mutants *uvrA* and *uvrC* was unaffected by the chloramphenicol pretreatment (Fig.1c). This is consistent with the early results of Hanawalt demonstrating that following a period of amino acid starvation, wild type but not repair deficient cells were more resistant to UV (13). Similarly, Tang and Smith found that liquid holding recovery was blocked in *uvr*$^-$mutants but not *recF*- cells (56). Common to both stationary phase cultures and chloramphenicol treated cultures, among other things, is that replication has been eliminated or greatly reduced. Therefore, one interpretation of these results is that the UV hypersensitivity associated with *recF* and *recR* cells is due to ongoing replication at the time DNA damage is introduced, rather than to a problem with the repair of the damage. Consistent with this, it has been shown that UV induced lesions are removed from DNA in *recF* cells with an efficiency comparable to that in wild type cells (44).

***recF* and *recR* show a delay in recovery of replication.** To investigate how replication may be affected in *recF* cells following UV irradiation, we examined the recovery of replication after UV irradiation in these cells. Using the incorporation of [^{3}H]thymine to monitor replication, we found that following a dose of 25 J/m^2, wild type cells exhibited a brief arrest before replication resumed at a rate comparable to that of unirradiated cultures.

For wild type cells, replication had fully recovered within one hour after irradiation (Fig.2a). In contrast, replication in *recF* and *recR* cells recovered more slowly and a significant lag was observed before any replication resumed.

This effect was also observed by density labeling the DNA with 5-bromodeoxyuridine to quantitate the amount of DNA replicated during the first hour following UV irradiation (Fig.2b). Cultures receiving either 25 J/m^2 or no irradiation were incubated in medium containing 5-bromodeoxyuridine for one hour so that any DNA synthesized during this period would be of a greater density than that of DNA synthesized before the irradiation. The denser, replicated DNA in each culture was separated from the rest of the DNA in an isopycnic alkaline CsCl gradient and quantitated. By this measure, the wild type cells had fully recovered within one hour. Thus, the amount of replicated DNA in the irradiated culture was nearly equivalent to the amount occurring in an unirradiated culture. Neither *recF* nor *recR* cells however, appeared to recover replication within this period. Interestingly, both *recBC* and *recD* cells appeared to recover fully within this time frame suggesting that the defect in recovering replication is specific to the *recF* pathway and not likely to be due to increased cell death occurring in these populations. However *recA*, which is known to be required for replication recovery, also showed a complete lack of replication following UV (18).

Increased degradation occurs at replication forks in *recF* and *recR* mutants. Because ongoing replication affected the UV survival of *recF*- cells, we speculated that the replication defect following UV may occur at existing replication forks rather than at new origins of replication initiation. We therefore examined the DNA at replication forks following UV. Exponentially growing [^{14}C]thymine prelabeled cultures were pulse labeled with [^{3}H]thymidine for 15 seconds to label the DNA at replication forks and then transferred to nonradioactive media just prior to irradiation. This facilitated the comparison of the amount of degradation occurring in the nascent strands of DNA at replication forks and in the genome overall.

In wild type cells, while very little degradation of the overall genomic DNA occurred, measurable degradation of the ^{3}H-DNA was seen before replication recovered (Fig.3). The evident accessibility of the DNA ends at replication forks to nuclease(s) suggests that the holoenzyme is often at least partially disrupted by DNA lesions, consistent with previous studies both *in vivo* and *in vitro* (12, 28). That complete degradation of the pulse labeled DNA does not occur before replication recovers is also consistent with the conclusions of others that cells have a mechanism to recover replication at existing forks rather than to abandon or degrade the partially replicated chromosomes (18, 45).

In principle, the precipitable ^{3}H pulse label should remain constant or decrease over time. However, in wild type cells we consistently saw an increase in precipitable ^{3}H label over time after replication had recovered. A comparison of the total ^{3}H within the cells with the acid precipitable ^{3}H label suggested that the effect is likely to be due to remaining intracellular pools of labeled nucleotides (data not shown). Excessive washing and chasing with non-radioactive nucleotides did not significantly reduce this phenomenon.

In contrast to the limited degradation seen in wildtype cells, the DNA at replication forks in *recF* and *recR* mutants was observed to undergo significantly more degradation for a greater extent of time. That degradation also appeared to be largely specific to growing fork regions (Fig.3). Genomic DNA was not extensively degraded in these cells. We interpret this to be consistent with the failure of *recF* and *recR* cells to recover DNA replication at the sites of existing DNA forks.

Unlike *recF* and *recR* cells, degradation occurring in *recA* cells was not specific to the growing fork DNA (Fig.3). This is consistent with Skarsted and Boye (1993) who reported that degradation of individual chromosomes occurs in *recA* cells in a *recD* dependent manner. In contrast to the *recA* degradation, we found that the degradation occurring at the growing forks was dependent upon *recJ*, a 5'-3' exonuclease belonging to the *recF* pathway and was not dependent upon *recD* (unpublished results).

Decreased ability to complete ongoing replication in *recF* and *recR* mutants. The preferential degradation of the growing fork DNA and the lag in recovery of replicative synthesis in *recF* and *recR* mutants imply that *recF* and *recR* function is required after replication disruption for the reassembly of a replication holoenzyme at a DNA fork. This suggests that DNA lesions create problems for *recF* mutants not because of a repair deficiency but rather because the lesions disrupt replication. If replication could be disrupted in the absence of DNA damage, a prediction would be that *recF* and *recR* cells, but not wild type cells, would show defects in the resumption of replication. For this reason, we looked at genomic replication in exponentially growing cells to see whether replication abnormalities could be detected in the absence of exogenous DNA damage. In our first approach, we used chloramphenicol or rifampicin to inhibit new rounds of DNA replication initiation and examined the cells' ability to complete the ongoing rounds of DNA synthesis as measured by incorporation of [^{3}H]thymine. By this criterion, both *recF* and *recR* cells were seen to accumulate less DNA than wild type cells (fig. 4a), suggesting that *recF*- and *recR*- cells are less able to complete ongoing rounds of DNA replication.

In an alternative approach, the chloramphenicol treated cells were analyzed by FACS after replication had ceased (3 hours after chloramphenicol treatment) to determine the chromosomal DNA content in individual cells. The *E.coli* chromosome contains one bidirectional origin of replication and because all origins within a cell initiate synchronously, normal cells complete replication with 2^n chromosomes per cell(51). Typically when exponentially growing cultures were treated, wild type cells completed replication with a distribution of 2, 4, or 8 chromosomes per cell (fig. 4b). However, when *recF* or *recR* cells were grown under the same conditions, significant deviations from this pattern were observed. In general, both mutants had fewer chromosomes per cell. In addition however, cells containing odd numbers of chromosomes were seen. The irregular chromosome number is consistent with the idea that a portion of the replication attempts failed to go to completion. It is likely that the disrupted replication forks, upon failing to restart are eventually degraded as has been reported to occur in *recA* cells(52). Genomic replication requires that two holoenzymes each replicate approximately 2.5 megabases of DNA to then meet at the other side of the genome. If the processivity of the polymerase were less than this extent, successful replication of the chromosome would require the restart of the replication fork. These results are consistent with a defect of *recF* and *recR* cells in restarting disrupted replication forks. Disruption in the absence of DNA damage may occur because DNA-bound proteins or DNA secondary structures block replication, a lack of DNA precursors required for the elongation inhibits progression, or perhaps the inherent processivity of the holoenzyme is sometimes exceeded during genomic replication.

It is important to point out that isogenic *thy*$^-$ strains were used in these studies to facilitate efficient radioactive labeling of the DNA. When we tested *thy*$^+$ strains of *recF*, *recR*, and *recO*, all were found to have normal chromosome profiles by FACS analysis (data not shown), suggesting that replication is disrupted more frequently in the *thy*- background. Consistent with this, the *thy*- mutation has been shown to affect the elongation rate of replication forks, presumably because the efficiency of processing and delivery of thymine nucleotides to the replication machinery is compromised in the auxotrophs, causing replication to stall more frequently(42, 59). By itself, the *thy*$^-$ mutation presumably does not introduce any exogenous damage. We postulate that by hindering the forward elongation reaction in *thy*$^-$ cells, the disassembly of a replication fork is favored by the slowed or stalled replication complex. Recently, it has also been shown that *thy*$^-$ strains of *E. coli* and *B. subtilis* are slightly more UV sensitive than wild type cells(29). Whether this is a replication dependent phenomenon has not been examined.

Discussion

We have shown that the UV hypersensitivity of *recF* and *recR* cells correlates with ongoing replication at the time of UV irradiation. The lag in replication recovery, in addition to the preferential degradation at existing growing forks, suggests that the hypersensitivity arises from a failure to resume replication from the DNA replication forks disrupted following irradiation. Our results support the idea that *recF* and *recR* gene functions are required for the reassembly of a holoenzyme at the site of a DNA replication fork. In the absence of *recF* or *recR*, reassembly does not occur and as a consequence, the DNA ends at the growing fork are accessible to more extensive degradation. These failed replication attempts can result in odd chromosome numbers and/or lethality when the cell is unable to recover or degrade the partially completed chromosomes.

While the problem of how replication deals with DNA damage has obvious relevance to both cell survival and mutagenesis, extremely little is known about it. Perhaps the simplest way one can imagine for a cell to deal with replication disruption would be to reassemble and reinitiate from the point of disruption. Presumably, in the case where a DNA lesion disrupts the replication holoenzyme, the disassembly and reassembly process would allow both the accessibility and time required for normal DNA repair processes to occur. Thus, the lack of any lesion blocking the resumption of DNA synthesis implies that there is no requirement for strand switching or recombination to occur during *recF* dependent reinitiation. Mechanistically, the reassembly reaction could be rather straight forward. RecF, and RecR may be involved in recognizing the replication fork structure and assembling a holoenzyme at this site much as the DnaA protein recognizes the structure created at *oriC* (figure5).

The well documented complexity of initiation from *oriC* demonstrates how critical the proper initiation of replication is for the cell. Through tight regulation of the time and location at which a replication fork is initiated, the cell ensures that upon division, each daughter cell will receive an equal and precise copy of the chromosome. Replication initiated either at the wrong time or at the wrong site(s) could be disastrous for the cell. Both thymineless death and stable DNA replication are phenomena that demonstrate the deleterious effects on viability caused by the loss of regulation in replication initiation (21, 38). Should a replication fork fall apart before reaching the terminus, the cell is faced with a dilemma. Simply abandoning the fork would likely create a lethal situation in which subsequent rounds of replication would only amplify the partial genomes and create problems of hyperrecombinagenic ends, gene dosage problems, as well as problems for chromosomal segregation. Survival will likely require that the cell either degrade these aborted attempts or ensure that they are completed. Under conditions in which no

exogenous source of DNA ends is introduced or generated within the cell, the structure shown in figure 5c should arise only when the holoenzyme is disrupted. If so, this would represent a legitimate substrate from which to initiate replication and still maintain the proper symmetry of replication forks per chromosome per cell.

Such a model implies that the UV sensitivity of *recF* is not due to a recombination defect. However, there exist abundant genetic and biochemical data demonstrating that recombination occurs in a *recF* dependent fashion. The literature suggests that recombination initiated via the *recF* pathway requires the presence of a 3' overhang DNA end and the participation of the RecA protein. These requirements for recombination may also provide insight as to how RecF may be functioning at replication forks in vivo.

The requirement for a 3' single stranded DNA end is inferred from the properties of the exonucleases which are associated with the *recF* pathway of recombination. RecJ is a 5'-3' exonuclease belonging to the *recF* pathway (31). Supressors which restore recombination to *recBC* mutants through activation of the *recF* pathway have thus far been shown to map to genes for other DNA exonucleases. SbcA and sbcB are generally thought to activate exoVIII, a 5'-'3 exonuclease, and to inactivate exoI, a 3'-5' exonuclease, respectively (17, 41). SbcC and sbcD suppressors are also thought to encode nuclease activities but their substrate specificities have not been defined (27). Thus, exonucleases involved in the *recF* pathway appear to process DNA ends so as to generate a 3' overhang.

A second requirement for *recF* recombination is the RecA protein. Biochemically, RecA is known to utilize an invasive 3' single stranded end to initiate the pairing of homologous DNA sequences (22, 23). In general, the biochemical and genetic evidence suggests that *recF*-mediated recombination is initiated by RecA using a 3' overhang to invade a homologous double stranded target sequence as shown (Fig. 5b). When one compares the structure generated by the RecA strand invasion to that of a disrupted replication fork, there is a striking similarity (Fig. 5c). Just as RecA is thought to catalyze the strand invasion of a 3' single stranded end into homologous duplex DNA, the holoenzyme, through the polymerization of the leading strand, catalyzes the invasion of a 3' single stranded end into homologous duplex DNA. In essence, the disrupted replication fork is the product of the reaction catalyzed by RecA. Based upon the same biochemical reaction, the RecA protein should promote the maintenance of this structure, should it begin to disassemble. Thus the substrate from which the cell reinitiates replication and the substrate believed to initiate *recF* recombination are structurally identical. It is then tempting to speculate that, since *recF* and *recR* are required to reinitiate replication from this substrate in vivo, as we have shown, the recombination that results from the *recF* pathway may occur when foreign DNA ends present in the cell are processed to mimic

those of a disrupted replication fork. In the case of recombination, the invading DNA has "fooled" the cell into believing that this is a legitimate substrate upon which to initiate replication.

The idea that a recombination deficiency is not responsible for the UV hypersensitivity in *recF* and *recR* mutants can also be inferred from genetic data on other genes in the *recF* pathway. *recJ* and *recQ* are also genes belonging to the *recF* pathway. Similar to *recF* and *recR*, these genes are required for recombination when the major pathway (*recBC*) is defective (30, 37). However, neither *recJ* nor *recQ* is hypersensitive to UV (30, 37). Thus, cells which should lack the ability to carry out recombination via the *recF* pathway are not necessarily hypersensitive to UV.

Replication has recently been shown to be required for other forms of recombination as well. The primosomal mutant, *priA* has been shown to be defective in both conjugational and transductional recombination. Interestingly, suppressor mutations that restore recombination in this background map to the *dnaC* gene (20, 48). The absolute requirement of replication for recombination to occur in these systems has led Kogoma to suggest that all recombination and double strand break repair in *E. coli* is carried out via replication (19). We believe that at the level of the chromosome, it is worthwhile to consider the possibility that many of the classically defined recombination proteins may function to maintain the chromosome without DNA strand exchange.

Our in vivo observations of *recF* and *recR* suggest that their association with the replication machinery goes beyond the level of genomic organization. We see that RecF and RecR are required to resume replication from a replication fork and that this can account for the UV hypersensitivity of these mutants. A general model is proposed for *recF* and *recR* function in which they participate to recognize a replication fork structure and reassemble a replication holoenzyme at this site. The model implies that the UV hypersensitivity is not due to a recombination defect but is still consistent with many of the recombinational phenotypes associated with the *recF* pathway.

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Figure 1. The UV hypersensitivity of *recF* and *recR* is dependent upon the replication state of the cell. (A) The survival of parental (BG)SR108, *recF*- (JE)HL219, and *recR*- cells (HC)HL220 is shown following UV irradiation at the indicated dose for both stationary phase (filled symbols) and exponentially growing cultures (open symbols). RecF- and *recR*- cells are less UV sensitive in stationary phase cultures. (B) The survival of parental (BG)SR108, *recF*- (JE)SR108F, and *recR*- cells (HC)SR108R, is shown following UV irradiation at the indicated dose for both cultures pretreated for 3 hours with chloramphenicol (filled symbols) and untreated exponentially growing cultures (open symbols). (C) Cells were treated as in (B) for *uvrA*- (R▷)HL758 and *uvrC*- cells(Q◁)HL763. Survival curves represent the average from at least three independent experiments except for *uvrA* and *uvrC* which were carried out once and twice respectively.

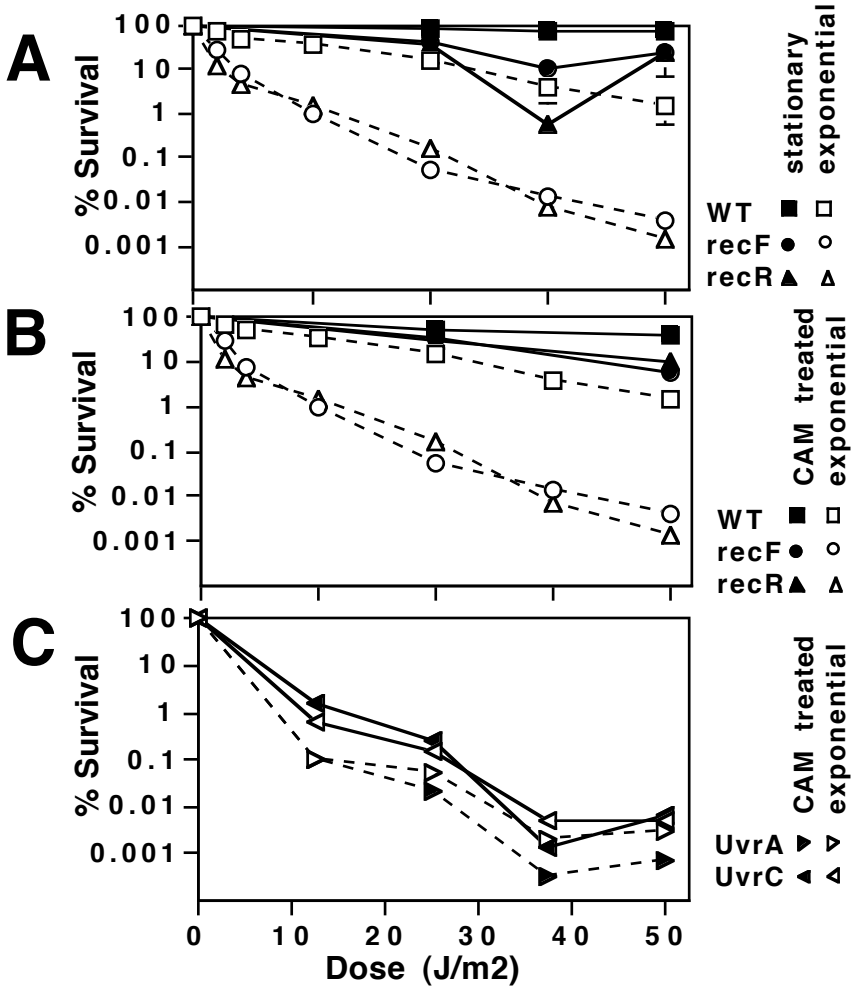


Figure 2. *recF-* and *recR-* cells show a delay in the recovery of DNA synthesis following UV irradiation. (A) Cells were prelabeled with [^{3}H]thymine. At time 0, half the culture was removed and given a dose of 25 J/m2(J) while the other half was left unirradiated(G). The relative increase of DNA as measured by ^{3}H incorporation is plotted. (B) The amount of replication occurring within one hour post irradiation was analyzed by alkaline CsCl density gradients. Cells prelabeled with [^{14}C]thymine were irradiated or not at time0. Cells were then filtered and grown in media containing 5-bromodeoxyuridine and [3H]thymine for one hour to density label replication occurring after time0. (G)^{14}C Prelabeled DNA, (E)^{3}H replicated DNA in unirradiated cultures, and (J)^{3}H replicated DNA in irradiated cultures.

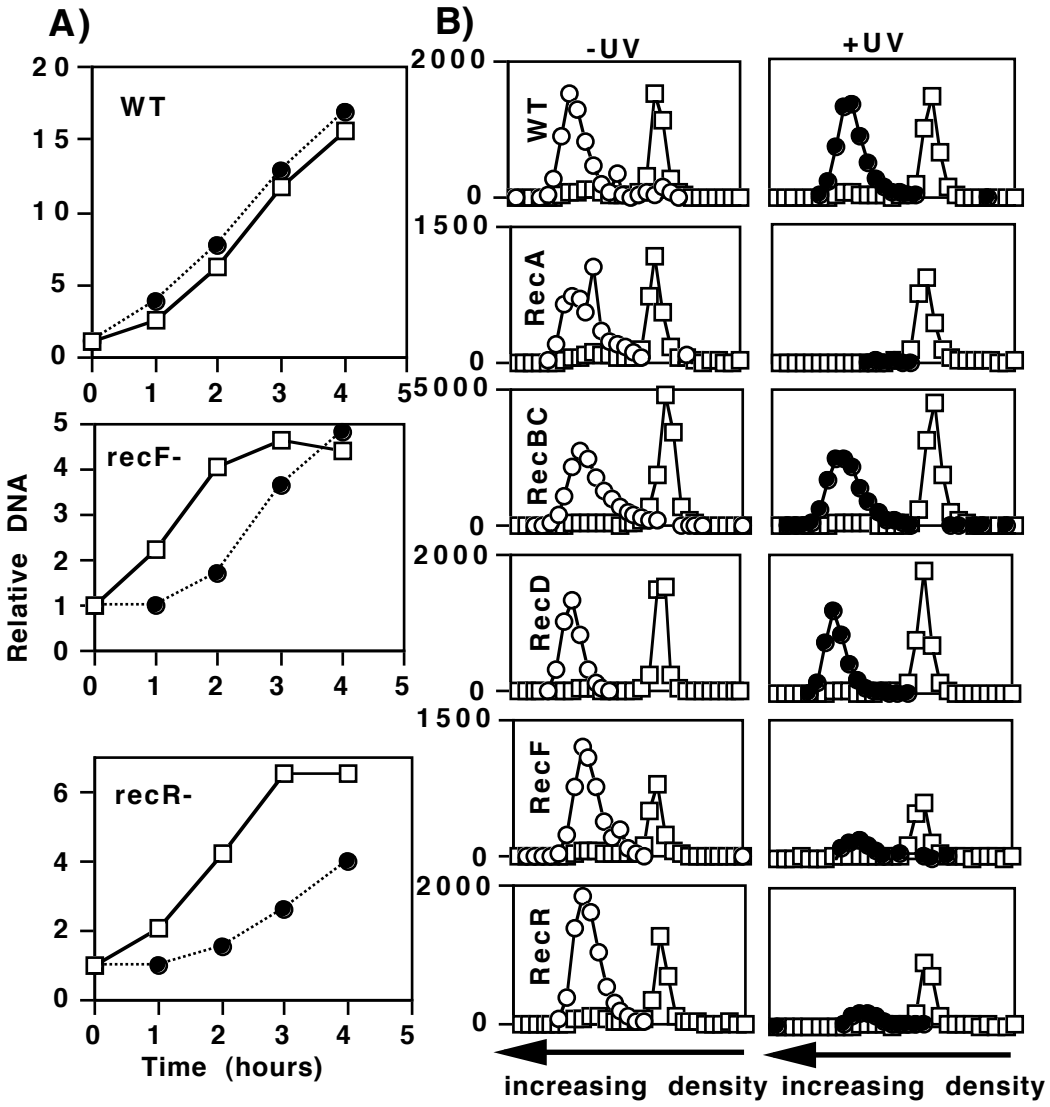


Figure 3. Increased degradation occurs at the growing fork following irradiation in *recF* and *recR* cells. [^{3}H]thymine was added to [^{14}C]thymine prelabeled cells for 30 seconds immediately before the cells were then filtered and irradiated with 25 J/m2 in nonlabeled medium. The fraction of the radioactive nucleotides remaining in the DNA is plotted over time. (BG)Parental cells, (JE)*recA*, (HC)*recF*, (PS)*recR*. Loss of ^{14}C genomic DNA (open symbols) can be compared to the loss of the ^{3}H DNA synthesized at the growing fork just prior to irradiation (filled symbols).

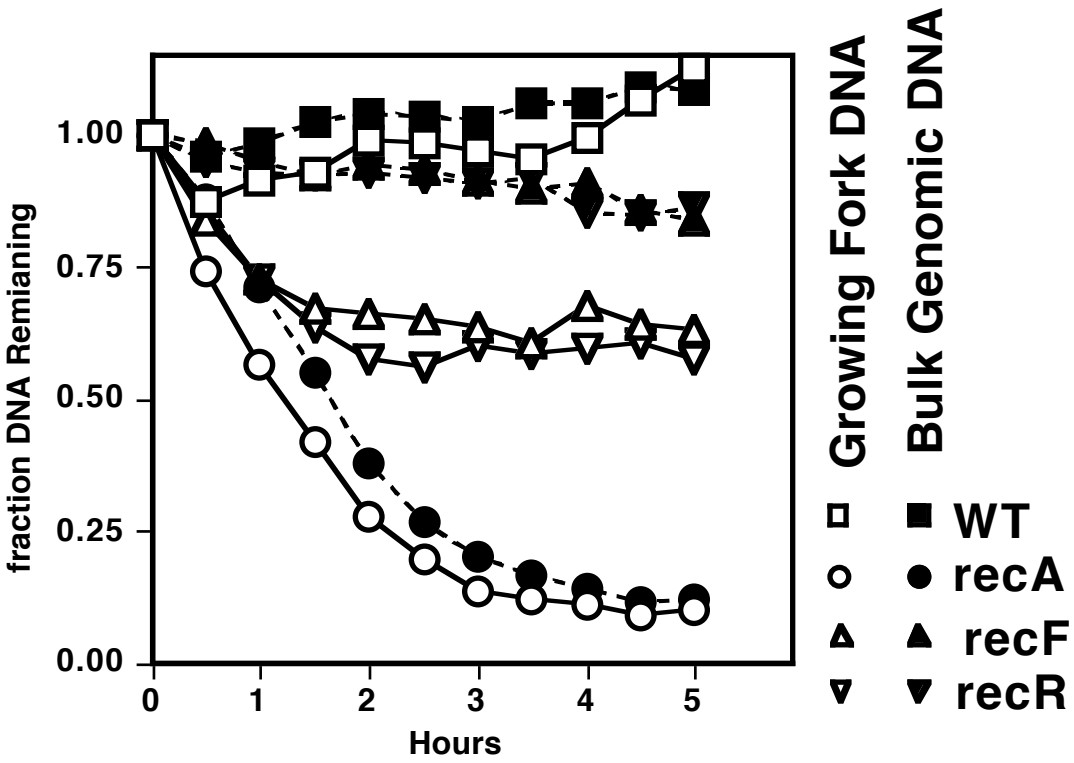


Figure 4. *recF*- and *recR*- cells are less able to complete ongoing rounds of DNA replication. (A) Chloramphenicol or rifampicin was added to cells prelabeled with [^{3}H]thymine and their ability to complete ongoing rounds of DNA synthesis as measured by net increase in DNA was analyzed.(B)Wild type, (J)*recF*, (H)*recR*. Chloramphenicol (solid line), Rifampicin (dashed line). (B) Chloramphenicol and cephalexin were added to cells prelabeled with [^{3}H]thymine and incubation continued for 3 hours. The cells were then analyzed by FACS to determine the DNA content per cell.

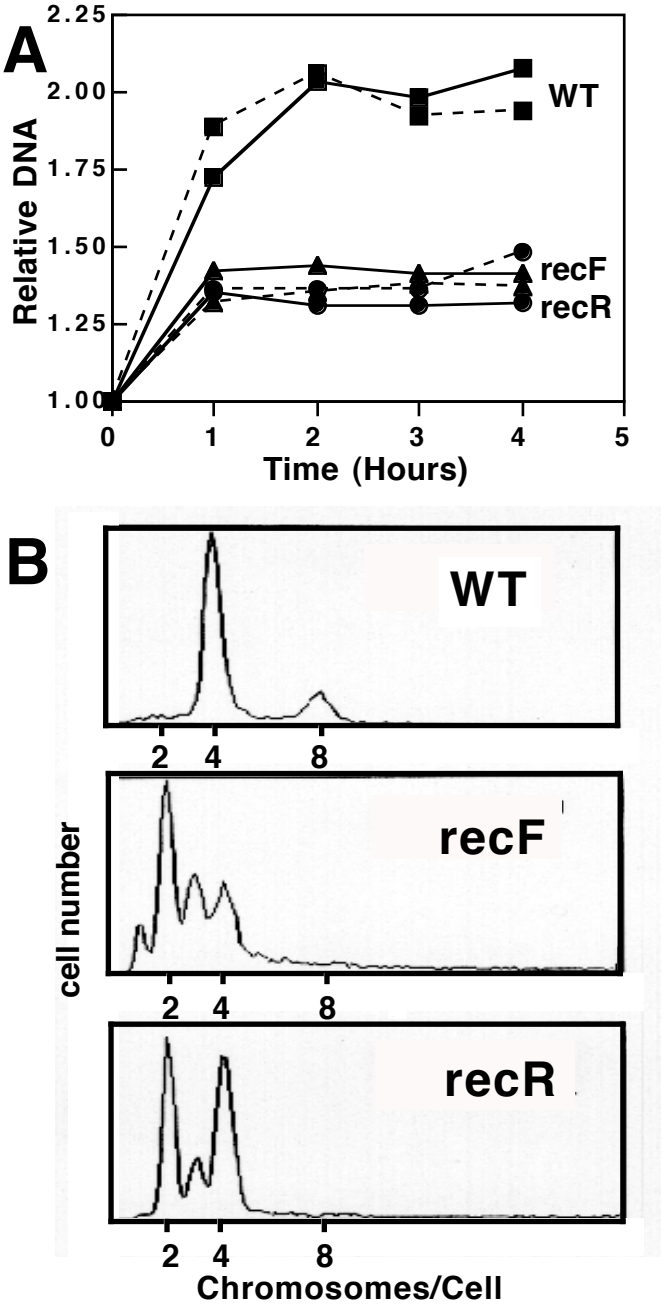
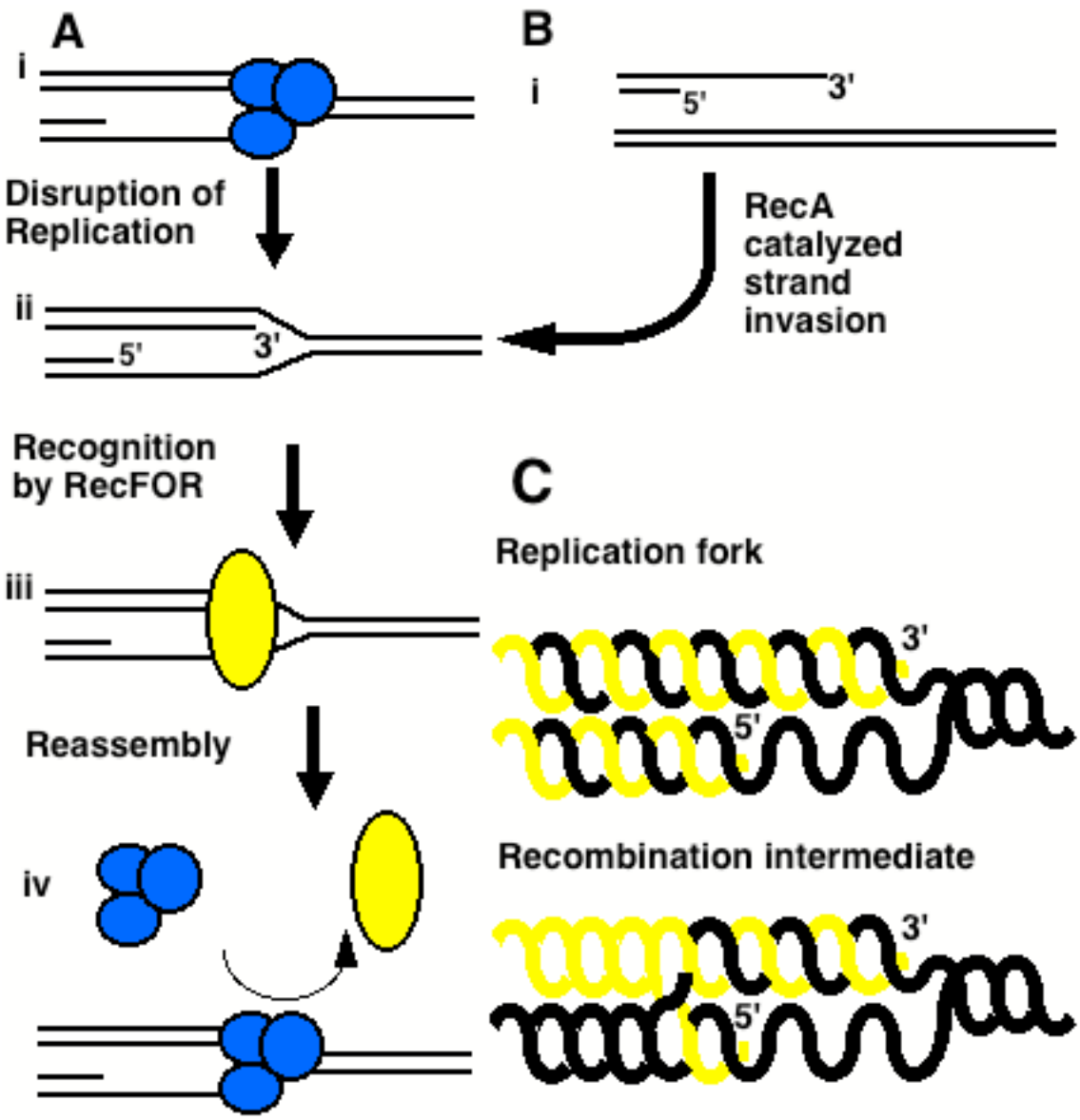


Figure 5. Model of *recF* function *in vivo*. The replication holoenzyme. *recF recO recR*. DNA synthesis occurs in a 5'-3' direction on both strands of duplex DNA. Thus, during semiconservative replication there exists a single stranded region near the replication fork on the lagging strand template which will vary in length depending upon where the last lagging strand primer exists (A,i). During genomic replication, if the holoenzyme were to fall off before the completion of replication, the replication fork would be expected to have a structure similar to that shown (ii). Polymerization of the leading strand will terminate with a 3' end inserted into the homologous double stranded template DNA. In the simplest model consistent with our results, RecF, RecO, and RecR would recognize this structure as a disrupted replication fork (iii), and facilitate the reassembly of a replication holoenzyme at this structure such that semiconservative DNA synthesis could resume (iv).

Such a function for the RecF proteins could also result in recombination when DNA ends are introduced into the system. DNA ends may be present when excessive damage has created strand breaks, when phage DNA has infected the cell, or when DNA has been transfected in artificially. In this situation, *recF* dependent recombination is observed to occur when exonucleases process the DNA ends to leave 3' overhangs (Bi). RecA, which is also required for *recF* recombination, is known to catalyze the strand invasion of 3' single stranded DNA into homologous duplex DNA. If this occurs, the structure created would again be a DNA strand terminating with a 3' end inserted into homologous duplex DNA as shown (ii). Comparing the resulting structures one finds that they are very similar(C), suggesting that the *recF* pathway proteins would also recognize this structure. Replication initiated from these DNA ends would incorporate the foreign DNA into the host and result in a recombination event. Such a role for *recA* in recombination suggests that in vivo, it may help maintain the replication fork following holoenzyme disruption.


A
i
Disruption of Replication
ii
5'
3'
B
i
5'
3'
RecA catalyzed strand invasion
Recognition by RecFOR
iii
Reassembly
iv
C
Replication fork
3'
5'
Recombination intermediate
3'
5'

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Chapter 3

Recovery of DNA replication in UV-irradiated *Escherichia coli* requires both excision repair and RecF protein function

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Abstract.

After UV doses that disrupt DNA replication, the recovery of replication at replication forks in *E. coli* requires a functional copy of the *recF* gene. In *recF* mutants, replication fails to recover and extensive degradation of the nascent DNA occurs, suggesting that *recF* function is needed to stabilize the disrupted replication forks and facilitate the process of recovery. We show here that the ability of *recF* to promote the recovery of replication requires that the disrupting lesions be removed. In the absence of excision repair, *recF*$^+$ cells protect the nascent DNA at replication forks, but replication does not resume. The classical view is that recombination proteins operate in pathways that are independent from DNA repair, and therefore the functions of Rec proteins have been studied in repair deficient cells. However, mutations in either *uvr* or *recF* result in failure to recover replication at UV doses from which wild type cells recover efficiently, suggesting that *recF* and excision repair contribute to a common pathway in the recovery of replication.

Introduction.

The *uvrA*, *uvrB*, and *uvrC* genes of *E. coli* are required for the incision and removal of UV-induced lesions from the DNA. *E. coli* strains mutated in any one of these genes are unable to remove these lesions and are extremely sensitive to UV irradiation (10, 39).

Other mutations which confer hypersensitivity to UV include those in the *recF* gene, originally identified as a gene required for conjugational or transductional recombination in *recBCsbcBC* mutants (15). In an otherwise wild type background, however, the *recF* mutants are fully proficient in recombination by these assays, though interestingly they remain hypersensitive to UV irradiation. *recO* and *recR* mutants were identified independently and are equivalent to *recF* mutants in their UV sensitivity and recombinational phenotypes when tested alone or in a *recF* background (21, 29). Together, these genes are commonly considered to operate in the *recF* pathway of recombination or repair (3, 24, 46).

RecF function appears to be tightly associated with DNA replication, in vivo. At the genomic level of organization, *recF* and *recR* are polycistronic with the *dnaN* and *dnaXZ* gene, respectively (9, 32). Both *dnaN* and *dnaXZ* encode core subunits of the replication holoenzyme. Additionally, a mutation in *priA*, a component of the primosome, has been shown to be lethal in combination with a *recF* mutation. Suppressors of this lethality map to the *dnaC* gene, yet another component of the replication machinery (40, 41).

A functional *recF* gene is implicated in several aberrant forms of replication, such as plasmid linear multimer formation, rifampicin-resistant plasmid replication, stable DNA replication, and thymineless death (20, 25, 27, 28, 31). While these processes are all abnormal and non-productive for cellular survival, they all involve extensive DNA replication.

The recovery of replication in UV-irradiated *E. coli* also requires a functional copy of the *recF* gene. In its absence, replication fails to recover and extensive degradation of the nascent DNA occurs (7). We hypothesized that the UV hypersensitivity of *recF* cells could be explained by a failure of these cells to recognize and resume replication from disrupted replication forks (7).

A role for RecF in the resumption of replication from disrupted replication forks could also explain how *recF* may promote recombination. Genetic and biochemical data suggest that RecF-mediated recombination utilizes a recombinational intermediate which mimics the structure of a disrupted replication fork. For recombination to occur in vivo, it requires a 3' single stranded overhang to be paired with homologous duplex DNA (1, 18, 22, 23, 26, 33). In the case of a disrupted replication fork, this identical structure is created by the leading strand of DNA synthesis that polymerizes an invading 3' DNA end into a homologous duplex template (7).

The ability of RecF to promote the resumption of replication from the site of disruption in UV irradiated cells may remain blocked by the replication-arresting lesions. If the resumption of replication requires that the arresting lesions must first be repaired, then one would predict that nucleotide excision repair should have a large effect on the resumption of replication. Indeed, the discovery of nucleotide excision repair followed from the characterization of UV sensitive bacterial mutants in which replication did not recover (42). In order to understand the mechanism of replication recovery more clearly, we have characterized the role of excision repair in the ability of RecF to promote the recovery of replication.

Materials and methods.

Bacterial Strains. SR108 is a *thyA36 deoC2* derivative of W3110. HL946 (SR108 *recF*332::Tn*3*), HL952 (SR108 *uvrA*::Tn*10*), HL925 (SR108 *uvrC*::Tn*10*) and HL1034 (SR108 *recA*::Tn*10*) were made by P1 transduction of the *recF*332::Tn*3*, *uvrA*::Tn*10*, *uvrC*::Tn*10* and Ç(*srlR-recA*)*306*::Tn*10* markers from strains HL556, HL758, HL765 and JC10289, respectively. The *recF*, *uvrA*, *uvrC* and *recA* phenotypes were checked by UV sensitivity.

Qualitative Survival Following UV. A fresh overnight culture was evenly applied onto an LB plate using a cotton swab and incubated at 37C for one hour. The plate was covered by a sheet of aluminum foil and placed under a 15-watt germicidal lamp (254 nm, 0.6 J/m^2/sec). The foil was progressively retracted following 20 J/m^2 exposures. The irradiated plate was then incubated at 37C for 8 hours and photographed.

Time course of replication recovery. Cells were grown in Davis media supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy media) containing 1.0 μCi/ml [^{3}H]thymine to an OD600 of 0.2 (approximately $3*10^8$ cells/ml) at which point half the culture received an incident dose of 25 J/m^2 (time 0). The amount of ^{3}H incorporated into the DNA was measured by averaging duplicate, 0.2ml samples precipitated in 5% cold trichloroacetic acid, and then collected on Whatman glass fiber filters.

Density labeling of replicated DNA. Cells were grown in DGCthy medium containing 0.2μCi/ml [^{14}C]thymine to an OD_{600} between 0.3 and 0.4 before harvesting by filtration and resuspending in DGC medium containing 10μg/ml 5-bromodeoxyuridine. Half the culture received 25 J/m2, each half received 0.5μCi/ml [^{3}H]thymine and was then incubated for 1 hour. 10 ml samples were placed in an equal volume of ice cold NET buffer (100mM NaCl, 10mM Tris pH8.0, 10mM EDTA), pelleted, and lysed in 0.4ml of 0.5M K_3PO_4 (pH 12.5) containing 40μl of 10% sarcosyl. The solution was then subjected to isopycnic alkaline CsCl gradient sedimentation as described (45). Thirty fractions were collected on Whatman#17 paper. The amount of ^{14}C and ^{3}H in each fraction was determined by scintillation counting.

Measurement of Global DNA Repair Cells were grown in DGCthy media containing 1.0 μCi/ml [^{3}H]thymine to an OD600 of 0.4 at which point cells were irradiated with a dose of 25 J/m2 in the defined medium and returned to the shaking, 37C water bath. 10 ml samples were removed at each time point and mixed with 2 volumes of ice cold NET. Cells were pelleted, resuspended in 0.5ml NET and 100 μg/ml RNase, and lysed by sonication in a Branson Sonifier. 10μl of 10mg/ml proteinase K and 10μl of 10% sarcosyl was added to the lysate and incubated for 1 hour at 65C. The DNA was extracted with phenol-chloroform and precipitated in 2.5M ammonium acetate and 2 volumes of ethanol. Purified DNA was resuspended in NET. The concentration of each sample was determined by fluorometry using Hoechst 33258 dye (2). The removal of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) from the DNA was measured by using an immunoassay (19). Following denaturation by boiling, 200 ng (CPDs) or 1 μg (6-4PPs) of each DNA sample was loaded in triplicate onto Hybond N+membrane, using a slot blot apparatus. The membrane was incubated for 2 h in the

presence of a mouse antibody against either CPDs (TDM-2) or 6-4PPs (64M-2) diluted 1:2000 in phosphate buffered saline (PBS) (antibodies were the generous gift of Toshio Mori (30)). Horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:5000 and detected with enhanced chemiluminescence (Amersham) and subsequent phosphorimager (Bio-Rad) analysis. Following detection, the amount of ^{3}H labeled DNA loaded in each slot was confirmed by scintillation counting.

DNA degradation following UV irradiation. Cells were grown in DGCthy medium containing 0.2μCi/ml [^{14}C]thymine to an OD_{600} between 0.3 and 0.4. Ten seconds before harvesting by filtration, 1μCi/ml [^{3}H]thymidine was added to the culture. Cells were resuspended in nonradioactive DGCthy media and irradiated with a dose of 25 J/m2 unless otherwise indicated. Approximately 10 and 20 seconds elapsed between resuspension and irradiation. The amount of ^{14}C and ^{3}H remaining in the DNA was measured as before (see above).

Results.

Replication recovery is inhibited in excision repair mutants and in *recF* mutants. The *recF* gene is generally considered to function independently of the *uvr* genes. However, the survival following moderate doses of UV requires that both genes be functional (figure1 and (35)). Previous studies revealed a dose dependent inhibition of replication in both excision deficient mutants and in *recF* mutants (35, 37, 42). To assess the contribution of excision repair and *recF* in the normal recovery process, we compared the recovery of replication in *uvr* and *recF* mutants to that in wild type cells.

Using the incorporation of [^{3}H]thymine to quantitate replication, we found that following UV irradiation with 25 J/m^2, the wild type cells exhibited a brief arrest of DNA synthesis before replication resumed at a rate comparable to that of unirradiated cells. However, when *recF* or *uvr* mutants were examined, the recovery of replication was either significantly delayed or did not occur (fig2A).

The inhibition of replication in *recF* and *uvr* mutants can also be observed by density labeling the DNA with 5-bromouracil to quantitate the amount of DNA replicated during the first hour after irradiation. Cultures receiving either 25 J/m^2 or no irradiation were incubated in medium containing 5-bromouracil (in place of thymine) for a period of 1 hour, so that any DNA replicated during this period would be of a greater density than the DNA synthesized before the time of irradiation. The denser, replicated DNA in each culture was separated from the rest of the DNA by centrifugation in an isopycnic alkaline CsCl gradient and quantitated. By this assay, irradiated wild type cells had replicated nearly as much DNA as the unirradiated control. However, neither the *recF* nor the *uvr* mutants appeared to replicate significant amounts of DNA within this period of time

(fig2B). In contrast, while *recBC* mutants are just as sensitive to UV as *recF* mutants, they recover replication normally following UV irradiation, suggesting that the failure to recover replication is not related to increased cell death in these populations (7).

The loss of replication recovery in either the *recF* or *uvr* mutants, at doses from which wild type cells completely recover, suggests that functional copies of *recF*, *uvrA*, and *uvrC* genes are required for the efficient recovery of replication. The results also suggest that in a wild type cell, *recF* function in replication recovery is greatly enhanced by the presence of excision repair.

***recF* mutants do not recover replication despite the repair of the UV lesions.** *recF* mutants have been reported to have an altered induction of the SOS response (48). SOS induction has been demonstrated to enhance the excision repair rate of the primary UV photoproducts (8). Thus the lack of recovery in *recF* mutants could be due to a failure to repair DNA lesions efficiently. However, Rothman and Clark (1977) found that the ability of UV-irradiated phage lambda to infect and form plaques was not significantly impaired in *recF* cells, inferring that excision repair was functional. Rothman then demonstrated using thin layer chromotography that dimers were excised in *recF* cells (34). To confirm this, we examined the rate of removal of the two primary DNA lesions produced by UV, the 6-4 photoproduct and the cyclobutane pyrimidine dimer, using monoclonal antibodies directed against each lesion.

In agreement with the results of Rothman and Clark (1977), we found that *recF* cells removed both lesions with rates comparable to those of wild type cells (figure 3A&B). *uvrA* mutants, as expected, did not remove significant amounts of either lesion. Though no difference in the rate of 6-4 photoproduct removal could be detected between wild type and *recF* cells, we observed a slight reduction in the removal rate of cyclobutane pyrimidine dimers in *recF* mutants, which may be a consequence of the delayed induction of the SOS response. However, repair was nearly complete within an hour in both wild type and *recF* mutants, suggesting that the lack of replication recovery in *recF* cells is not due to a failure to remove lesions from the template.

Nascent strand degradation at the replication fork occurs following replication disruption. The failure to recover replication in UV irradiated *recF* mutants is associated with the extensive loss of nascent DNA, made just prior to irradiation. Since replication also fails to recover in *uvr* mutants, we examined the degradation pattern in these mutants to determine whether their phenotype was similar to that of the *recF* mutants. Exponentially growing, [^{14}C]thymine prelabeled cultures were pulse labeled with [^{3}H]thymidine for 10 seconds to label the DNA at replication forks, and then transferred to nonradioactive medium just prior to irradiation. The ^{14}C prelabel

allowed us to compare the degradation occurring in the overall genome to that of the ^{3}H-labeled DNA made at replication forks just prior to UV irradiation.

Wild type cells degraded very little of their overall genomic DNA following irradiation. However, the nascent DNA exhibited moderate degradation at times prior to the recovery of replication, as determined above. The increase in ^{3}H after 60 minutes is probably due to intracellular pools of [^{3}H]thymidine incorporated following recovery which we were unable to wash out (data not shown). In contrast to wild type cells, the *recF* mutant degraded approximately half of the nascent DNA. Similar to the wild type cells, however, the degradation in *recF* cells was localized primarily to the replication fork DNA and very little degradation of the genome overall was detected (figure 4A).

In contrast to the *recF* mutants, the nascent strand degradation in the *uvr* mutants was limited to approximately the extent and duration seen in wild type cells (figure 4B). This result is interesting because, although neither *uvr* or *recF* mutants recovered replication, the *uvr* mutants did not display the extensive nascent strand degradation associated with *recF* deficiency. The absence of the *uvr* proteins however, did not seem to prevent the disruption of replication since degradation still occured in the *uvr* mutants. In addition, a *recF uvrA* double mutant exhibited the same extensive nascent DNA loss as did the *recF* single mutant (figure 4B and 4C).

Thus, while replication disruption appears to occur in wild type, *uvr* and *recF* cells as evidenced by the loss of nascent strand DNA following UV irradiation, only *recF* cells fail to recognize and protect the nascent strands from extensive degradation. The failure to recover replication in *uvr* mutants is not due to a failure to recognize and protect the nascent strands of the disrupted DNA fork.

Replication is only partially inhibited at low UV doses. Previous studies that have focused on the recombinagenic pathways of *E.coli* have examined post irradiation replication in either *recF* or *uvr* mutants at lower doses of UV (11-13, 37, 38). Since we found that replication is significantly inhibited following UV, we examined replication in these mutants at the lower doses used in other studies.

The amount of replication occurring post irradiation was quantitated as before by incubating irradiated cultures in 5-bromouracil to density label any DNA replicated within a one hour incubation period. The denser, newly replicated DNA was then separated in an isopycnic alkaline CsCl gradient and the amount replicated after various doses were compared.

Consistent with the results of previous studies (36, 37, 42), we found that replication was only partially inhibited at the lower doses. In either mutant, the inhibition of replication increased as the UV dose increased and the level of inhibition between the

uvrA and *recF* mutants was roughly comparable at a given dose (figure 5A and B). However, the fact that wildtype cells completely recover replication at doses which totally inhibit recovery in either mutant suggests that the resumption of DNA synthesis in wild type cells is dependent upon both gene products (figure2B).

In contrast to *recF* and *uvrA* mutants, the inhibition of replication occurs at much lower doses in *recA* cells (figure 5C). In addition, the *recA* cells degrade eighty to ninety percent of both the nascent and genomic DNA following even a low UV dose (7). The DNA degradation which occurs in *recA* cells has been shown to progress back from disrupted replication forks and does not occur in nonreplicating cultures (16). Since replication is disrupted at these low fluences in *recA* cells, it is unlikely that the partial inhibition seen in *recF* and *uvrA* mutants is due to a nonuniform exposure of the cell population to UV.

Discussion.

Following UV irradiation, *recF* and *uvr* mutants fail to recover replication at doses from which wild type cells recover efficiently. In *recF* cells, the DNA lesions are removed but the nascent strands of the disrupted replication fork are not protected and undergo more extensive degradation. In *uvr* mutants, the nascent strands are recognized and protected, but the recovery of replication remains blocked because the UV lesions are not removed. The data strongly suggest that in wild type cells, both RecF and excision repair operate in a common pathway of replication recovery.

We believe that the data are most consistent with the idea that following the disruption of replication by UV irradiation, *recF* function is required for the resumption of DNA synthesis from the disrupted replication forks following the removal of the UV lesions by excision repair (figure 6). The disruption of replication as evidenced by the transient arrest of DNA synthesis and loss of nascent DNA presumably allows both the time and accessibility required for excision repair to occur.

Because recombination proteins are usually considered to function independently from the process of nucleotide excision repair, previous models of RecF function have focused on how replication deals with lesions that arrest replication but which cannot be repaired. A large body of work in *uvr* mutants has demonstrated that following UV irradiation, the limited replication that occurs in the absence of excision repair is accompanied by significant amounts of *recF*-dependent strand exchange (11, 13, 37, 38). It has been proposed that in this case the DNA replication machinery can resume downstream of DNA lesions, creating the single strand gaps which are later "repaired" through *recF* dependent strand exchanges with sister chromosomes, a process termed post replication recombinational repair, or daughter strand gap repair (14).

Both the model presented in figure 6 and classical post replication repair models suggest that replication is disrupted and then resumes upon encountering UV lesions. The data presented here suggest that excision repair plays a large role in the ability to resume replication in wild type cells. As presented in figure 6, if excision repair occurs following disruption, replication may simply resume from the site of disruption rather than initiating replication from a new site downstream. The lack of replication recovery in UV-irradiated *recF* mutants, despite the proficient overall repair of the genome would suggest that, following disruption, replication does not efficiently resume downstream of disrupting lesions. If it did, one might expect *recF* to have wild type levels of replication recovery, but simply leave a gap(s) at the site(s) of disruption. Further, the fact that the nascent DNA is accessible to nucleases indicates that the region is not hidden by a stalled replication complex and implies that the region may also be accessible to repair enzymes. However, we cannot exclude more complex models in which replication reinitiates downstream of the lesion but then arrests again until the required steps of both recombination and repair have been completed.

The partial recovery which occurs in *uvr* and *recF* mutants following low doses of UV may highlight the conditions which promote recombination. However it may not represent the predominant mechanism of recovery in wild type cells since the wild type cells remain unaffected under these conditions while a significant reduction in both replication recovery and cell survival occurs in either mutant. That replication is not completely inhibited at low doses in these mutants could suggest that a class of lesions (such as those on the lagging strand template) do not disrupt replication or that these mutants retain a limited ability to bypass lesions.

The general view that the recombination function is independent from excision repair derives from early studies demonstrating that a *recA uvrA* double mutant was more sensitive to UV irradiation than either single mutant (17). However, wild type cells survive irradiations producing thousands of lesions per genome, whereas a mutation in either *uvrA* or *recA* reduces the lethal dose to less than fifty lesions per genome with more than 99.9% of cells losing viability before any cell death can be detected in wild type cells (17). The extreme hypersensitivity of either a *uvrA* or *recA* mutant would suggest that the majority of the survival and recovery occurring in wild type cells requires that both genes be functional. Similar to mutations inactivating *recA*, *recF* mutations also increase the sensitivity of *uvr* strains (35). However, as is the case with *recA*, the increase in hypersensitivity due to the addition of a *recF* mutation represents an almost insignificant portion of the lethality observed in either *recF* or *uvr* mutants when compared to the survival of wild type cells (figure1).

Other studies have also suggested a link between recombination genes and excision repair. The phenomenon termed "long patch excision repair" documented a similar dependency on both the *uvr* proteins and *recF* (4-6). Following UV irradiation, the size distribution of the DNA repair "patches" was found to be bimodal. At early times, short "patches" representing normal excision repair were the predominant species generated. However at the time that replication was seen to recover, longer "patches" of 1500bp and >9000bp in length were found. These "patches", which correspond in both size and ratio to those predicted for lagging and leading strand DNA synthesis, respectively, have been shown by two dimensional gel analysis to be localized at DNA replication forks (4). It is tempting to speculate that these *uvr*, *recF*-dependent patches may in fact represent the resumption of chromosomal replication following removal of the disrupting lesions.

The biochemical activity of RecF in the initiation of replication remains unknown. RecF may serve a largely structural role. This possibility is supported by observations that RecA filaments dissociate upon encountering DNA ends. In vitro, combinations of the RecFOR proteins function by stabilizing RecA filaments at DNA ends and limit the length of filaments extending into duplex DNA (43, 44, 47). The biochemical reaction of reassembling the replication fork structure is identical, in principle, to the mechanism by which RecA is thought to promote homologous strand pairing. The RecFOR proteins function through stabilizing the RecA filaments which maintain the replication fork structure following disruption. Alternatively, the RecF protein may play a more active role in the reestablishment of the replication machinery at these sites. This later possibility is attractive considering the genomic organization and genetic associations of the *recF* pathway with replication proteins as outlined in the introduction. It would prove interesting if these associations extended to direct biochemical interactions between the DNA and replication proteins as well.

Although the cellular role of recombination proteins is tightly associated with the replication of the chromosome, recombination proteins are generally studied independently from the process of replication. Replication is able to duplicate the genome in a semiconservative fashion, without alteration, generation after generation. The fact that many of the *rec* mutants in *E.coli* appear compromised in this ability suggests that these proteins contribute to the semiconservative duplication of the chromosome. Recombination events, ie. strand exchanges, occur at a very high cost to the organism and in higher organisms, they are intimately associated with genomic instability and a progression towards cancer. The requirement of strand pairing activities to accurately resume replication from disrupted replication forks may be the reason cells

endure this cost. Strand exchange may be a minor, perhaps inappropriate resolution of the strand reassembly process that is required following disruption. Genetic analysis however, whether scoring cancer in humans or an auxotrophic marker in *E. coli*, only reflects the exchanges, rather than the normal events that maintain the integrity of the genome.

Acknowledgements.

This paper is dedicated to the memory of Tokio Kogoma. His work, comments, and insights have significantly contributed to the present work and will be missed in the future.

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Figure 1. The survival of wild type, *uvrA*, and *recF* strains following UV irradiation with the indicated dose.

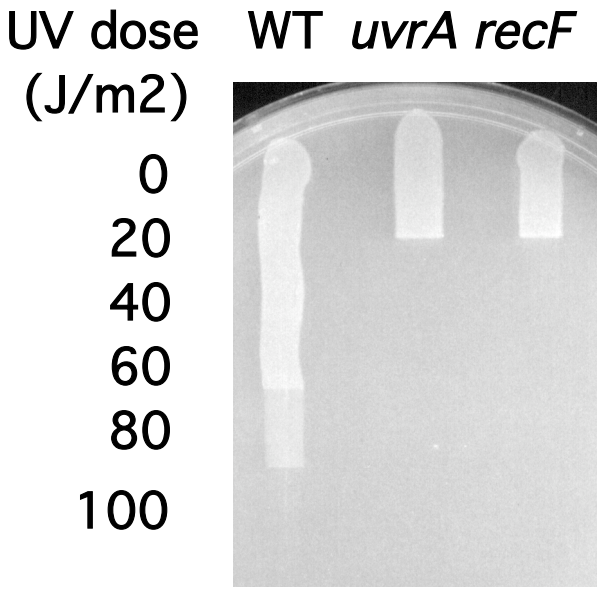


Figure 2. *recF* and *uvr* mutants show a delay in the recovery of DNA synthesis following UV irradiation. (A) Cells were prelabeled with [^{3}H]thymine. At time 0, half the culture was removed and given a dose of 25 J/m2 (filled symbols) while the other half was left unirradiated (open symbols). The relative increase of DNA as measured by ^{3}H incorporation is plotted. (BG)wild type (JE)*recF*$^{-}$ (HC)*uvrA* (PS)*uvrC*. The initial ^{3}H was between 1000 and 1500cpm for all strains. (B) The amount of replication occurring within one hour post irradiation was analyzed by alkaline CsCl density gradients. Cells prelabeled with [^{14}C]thymine were irradiated, or not, filtered, and grown in medium containing 5-bromodeoxyuridine and [3H]thymine for one hour to density label replication occurring this time period. (G)^{14}C Prelabeled DNA, (E)^{3}H replicated DNA in unirradiated cultures, and (J)^{3}H replicated DNA in irradiated cultures. The range of the peak fraction of ^{3}H in unirradiated cultures was between 58000 and 91000 cpm for all strains. The range of the peak fraction of ^{14}C was between 900 and 2100 cpm in all cases. The ratio of the maximum value between the ^{3}H axis and ^{14}C axis is held constant in all graphs.

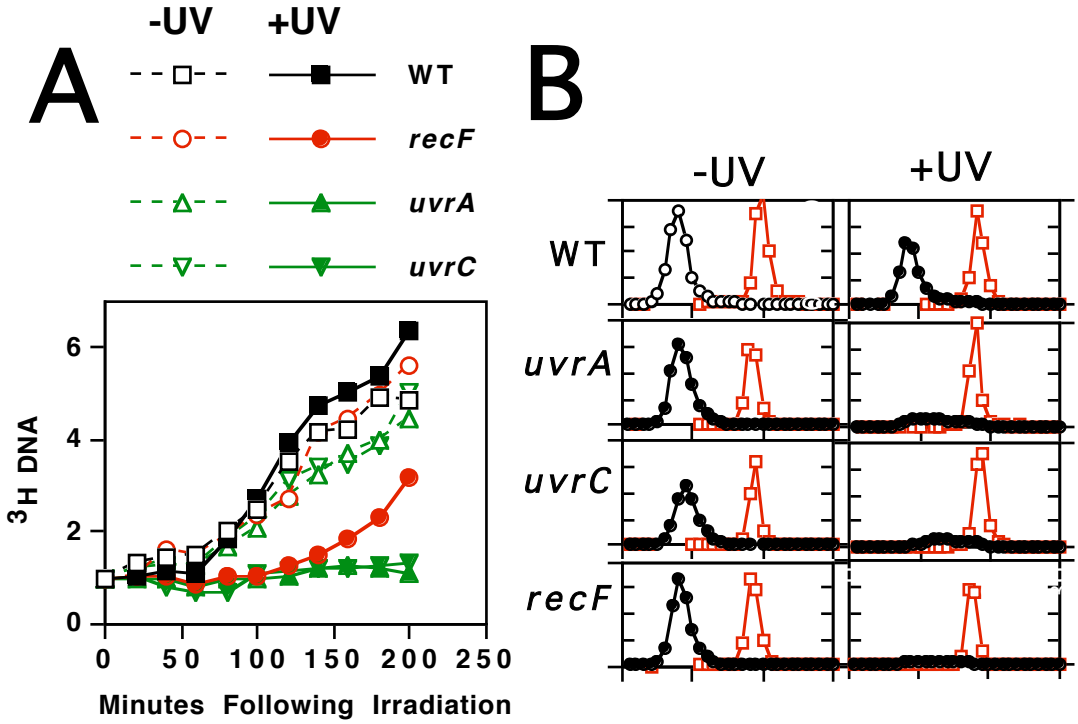


Figure 3. RecF removes UV lesions with kinetics that are comparable to those of wild type cells. Monoclonal antibodies specific for (A) CPDs and (B) 6-4PPs were used to assay lesion in DNA isolated at the indicated times following irradiation with 25J/m^2. (G) Wild type cells, (E) *recF* cells, and (C) *uvrA* cells. Points represent the average of two independent experiments each slotted in triplicate. A representative time course for each strain is shown next to each graph.

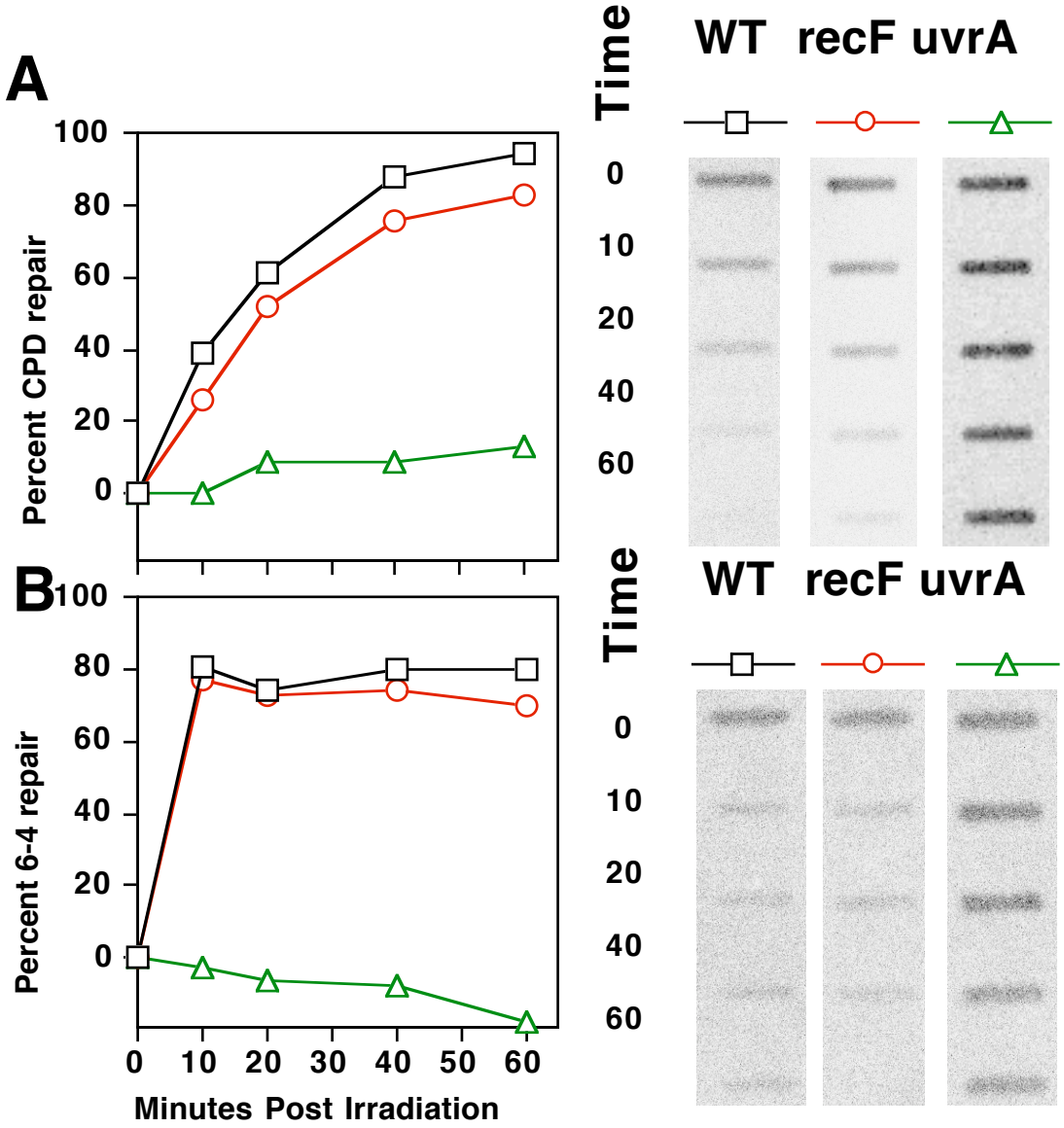


Figure 4. Following irradiation, increased degradation occurs at the growing fork in *recF* mutants but not *uvr* mutants. [^{3}H]thymine was added to [^{14}C]thymine prelabeled cells for 10-15 seconds immediately before the cells were filtered and irradiated with 25 J/m2 in nonlabeled medium. The fraction of the radioactivity remaining in the DNA is plotted against time. Loss of ^{14}C genomic DNA (open symbols) can be compared to the loss of the ^{3}H DNA synthesized at the growing fork just prior to irradiation (filled symbols). (A) (BG)wild type; (JE)*recF* cells. (B) (BG)wild type; (HC)*uvrA*; (PS)*uvrC* cells. (C) (FA)*recFuvrA;* (JE)*recF* ; (HC)*uvrA* cells. The range of the initial ^{14}C was between 900 and 1200 cpm and the initial ^{3}H was between 5800 and 10000 cpm in all cases.

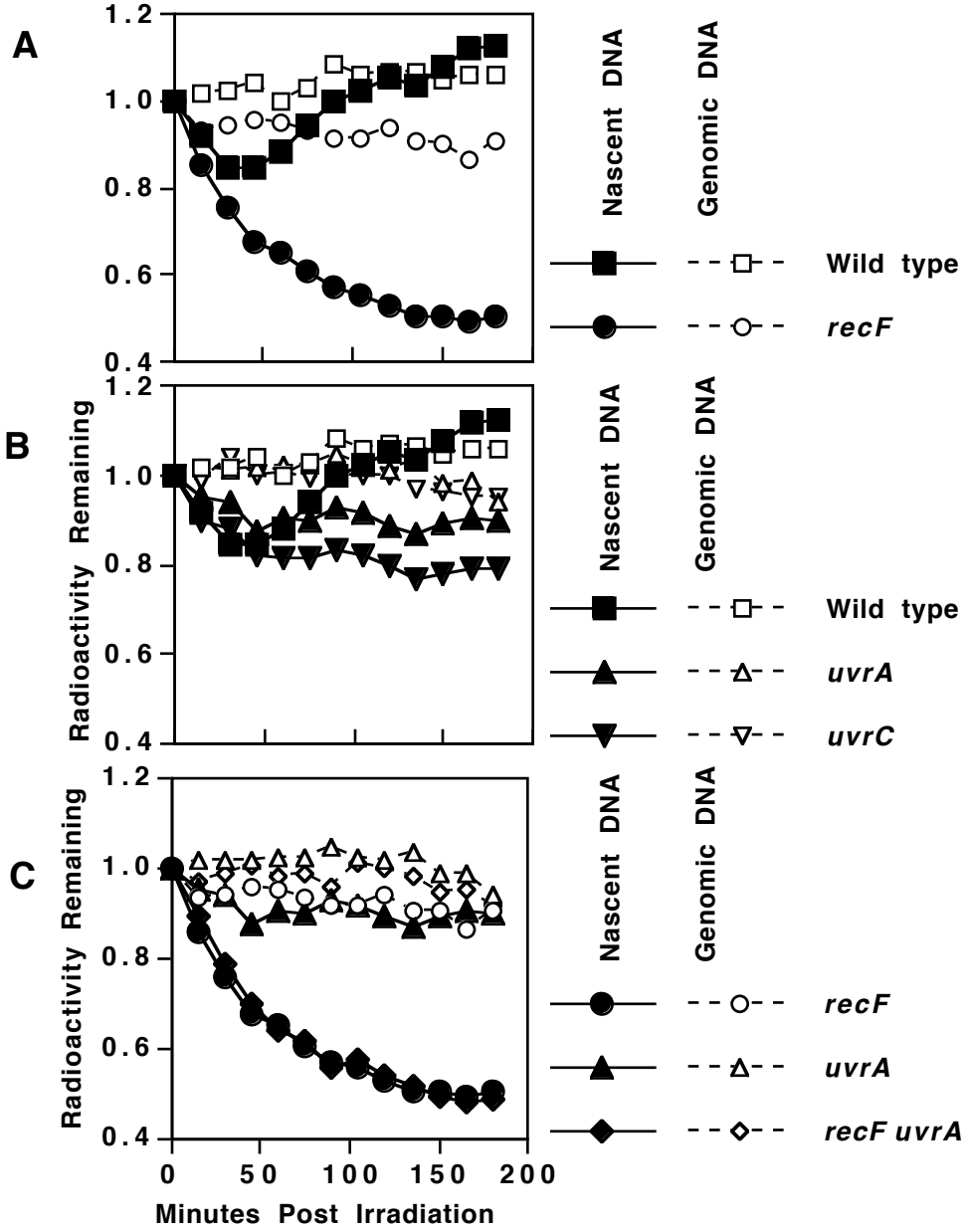


Figure 5. Replication is only partially inhibited after low doses of UV irradiation. The amount of replication occurring within one hour post irradiation at various doses was analyzed by alkaline CsCl density gradients. A single, [^{14}C]thymine prelabeled culture was filtered and placed in medium containing 5-bromodeoxyuracil and [3H]thymine. 10ml aliquots were immediately irradiated with the indicated dose. Cells were allowed to recover in a 37C shaking water bath for one hour to density label any replication occurring after irradiation. (G)^{14}C Prelabeled DNA, (E)^{3}H replicated DNA in unirradiated cultures, and (J)^{3}H replicated DNA in irradiated cultures. The range of the peak fraction of ^{3}H in unirradiated cultures was between 16000 and 47000 cpm for all strains. The range of the peak fraction of ^{14}C was between 900 and 4100 cpm in all cases. The range of the peak fraction of ^{14}C was between 900 and 2100 cpm in all cases. The ratio of the maximum value between the ^{3}H axis and ^{14}C axis is held constant in all graphs.

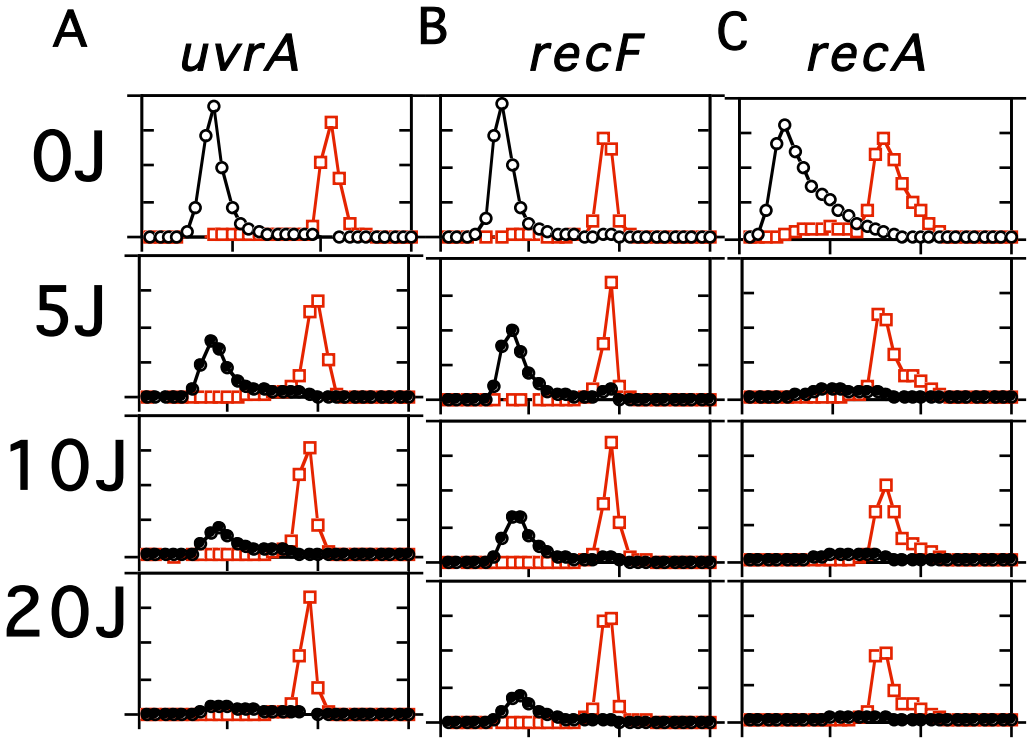
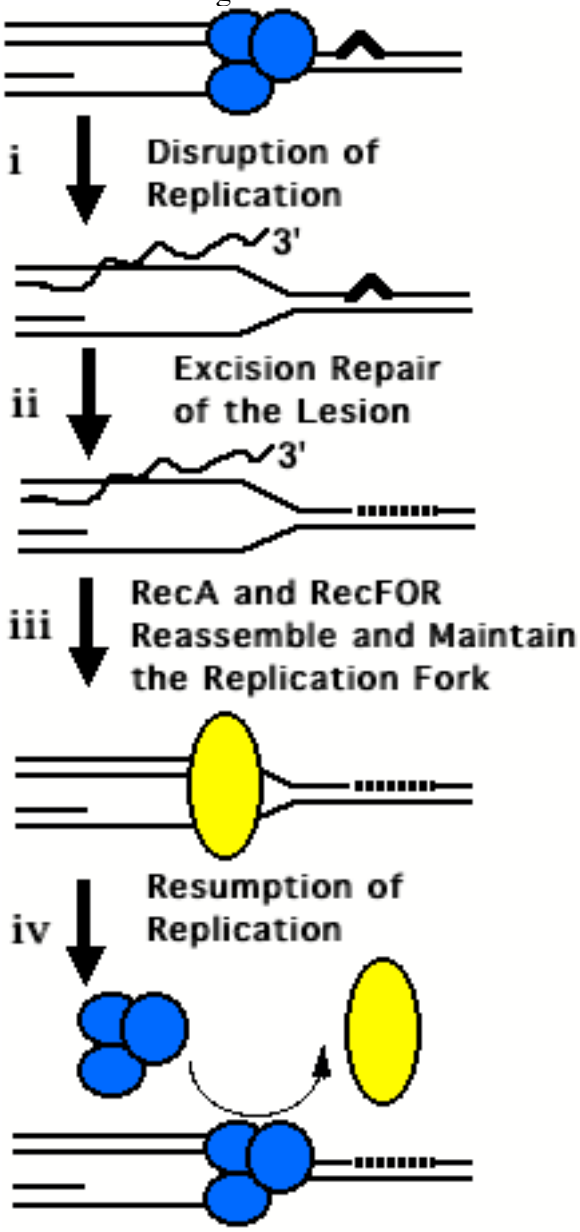


Figure 6. Model of replicational recovery. Replication is disrupted by a UV lesion in the DNA (i). The excision repair proteins then remove the blocking lesions from the parental DNA template (ii). However because the replication fork has been disrupted, resumption of replication requires that strand pairing/exchange proteins (RecA, RecFOR) are needed to reassemble and maintain the strands of the replication fork (iii) until redelivery to the replication machinery can occur (iv). In such a model, the recombinagenic activities of the RecA and RecF proteins function exactly as they have been characterized biochemically. In vivo however, it suggests that these enzymes are required to re-pair the strands of the replication fork as they were before the disruption event occurred, rather than pairing them with other homologous strands.



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Chapter 4

RecQ and RecJ Process Blocked Replication Forks Prior to the Resumption of Replication in UV-Irradiated *Escherichia coli*

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Abstract: The accurate recovery of replication following DNA damage and repair is critical to maintaining genomic integrity. In *Escherichia coli*, the recovery of replication following UV-induced DNA damage is dependent upon several proteins in the *recF* pathway, including RecF, RecO, and RecR. Two other *recF* pathway proteins, the RecQ helicase and the RecJ exonuclease, have been shown to affect the sites and frequencies at which illegitimate rearrangements occur following UV-induced DNA damage, suggesting that they also may function during the recovery of replication. We show here that RecQ and RecJ process the nascent DNA at blocked replication forks prior to the resumption of DNA synthesis. The processing involves selective degradation of the nascent lagging DNA strand and it requires both RecQ and RecJ. We suggest that this processing may serve to lengthen the substrate that can be recognized and stabilized by the RecA protein at the replication fork, thereby helping to assure the accurate recovery of replication after the obstructing lesion has been repaired.

key words: replication, DNA repair, *recJ*, *recQ*, *UV irradiation*

Introduction.

All cells must faithfully replicate their genomes to reproduce. However, DNA damage that blocks replication can lead to a loss of genomic stability, mutations, or cell death if not repaired. Despite the importance of the process by which replication recovers, the cellular mechanism(s) by which this occurs in DNA repair proficeint cells remains largely uncharacterized.

Irradiation of cells with near UV light induces lesions in the DNA which block replication. In *E. coli*, replication is transiently inhibited following a moderate dose of UV irradiation, but it efficiently recovers following the removal of the UV-induced lesions (7, 46, 47). The efficient recovery of replication in wild type cells is accompanied by the partial degradation of the nascent DNA at the replication fork prior to the resumption of DNA synthesis (6, 7, 18). However, it is not known whether this degradation is required for, or contributes in any way to the normal recovery process.

The resumption of replication following UV-induced DNA damage is largely dependent upon the removal of the lesions by nucleotide excision repair (7, 47). However, a large body of work with repair deficient mutants has documented that UV-irradiation can lead to recombination events when replication forks encounter DNA damage which cannot be repaired (11, 12, 43, 44). In these mutants, the recovery of replication is severely inhibited and results in loss of semiconservative replication, high

frequencies of chromosomal exchanges, and extensive cell death (6, 7, 11, 44, 47). In contrast, these recombination events are predominantly supressed in normal, repair proficient cells, in which the survival is greatly enhanced and the recovery of replication is much more efficient, suggesting that the normal mechanism of recovery may be quite different from that observed in repair deficient mutants (6, 7).

In addition to removal of the lesions however, the recovery of replication also requires the function of RecA and the *recF* pathway proteins (20, 51). Historically, because most of these proteins were identified through recombination assays, their functions have been assumed to be specific to recombination. However, in the presence of DNA damage, these genes are also required for genomic replication to occur (5, 20)(Clark and Margulies, 1965; Courcelle, et al., 1997; Courcelle, et al., 1998 Horii and Clark, 1973; Howard-Flanders, et al., 1969). Whereas these genes were identified because of their roles in the creation of recombinant progeny, genomic replication in bacteria normally creates clone cells which have identical genomes. The daughter genomes are generated through a processive, semiconservative replication of the parental genomic template (replicated regions which have undergone strand exchanges would not be semiconservative). The general lack of recombination which is observed during replication in asexual reproducive cycles suggests that although necessary for recombinational processes to occur, these *rec* genes may also be required for a different purpose during genomic replication.

One alternative function is suggested by several studies which demonstrate that these gene products are needed to stabilize and maintain the strands of the ongoing replication forks when they become blocked by DNA damage, rather than to promote DNA strand exchanges as occurs during recombination. The association with replication forks was initially inferred from observations that, although UV-induced lesions are removed from the DNA in *rec* mutants at rates which are similar to those in wild type cells, replication does not recover (4, 7, 42). Additionally, in *recA* mutants, it has been shown that when replication encounters a UV-lesion, a rapid and eventually complete degradation of the entire genome occurs (21) (unpublished observations). However, in either the presence of RecA or the absence of replication, the genomic DNA remains protected (21) (unpublished observations). Furthermore, the degradation which occurs in the absence of RecA has been shown to initiate at the blocked replication forks and then regress back from these points (21). These observations are all consistent with the idea that RecA function is required to stabilize and protect the strands of the replication forks which are blocked by DNA damage.

Cells with mutations that inactivate *recF*, *recO*, or *recR* also fail to recover replication following UV irradiation (6, 20, 24, 33). Following irradiation of these mutants, the genomic DNA remains largely intact. However, extensive degradation of the nascent DNA occurs, suggesting that the *recF* deficiency relates more specifically to an inability to recognize or resume replication from the blocked replication forks. In UV-irradiated wild type cells, the nascent DNA degradation is limited and occurs prior to the time at which replication recovers, suggesting that this degradation may be a normal part of the recovery process (6, 7). These observations have led to our proposal that a primary role of RecA and the *recF* pathway proteins during the asexual replication of the bacterial genome is to maintain blocked replication forks until replication can resume after the DNA damage has been repaired (6, 7). Purified RecA protein has been shown to bind single stranded DNA and to pair it with homologous duplex DNA, an activity which is thought to be critical in bringing together homologous pieces of DNA during recombination (for reviews see (25, 41, 49)). However during genomic replication, this same enzymatic activity could also function to protect and maintain the pairing between the strands of the replication fork until the block to replication has been removed.

Both RecQ (a 3'-5' DNA helicase) and RecJ (a 5'-3' single strand specific exonuclease) belong to the *recF* pathway, although unlike RecF, the absence of these proteins does not prevent the recovery of replication (31, 32, 38). However, mutations which inactivate RecJ have been shown to alter the sites and frequency of illegitimate rearrangements following DNA damage (52). *recQ* mutants have been shown to *increase* the frequency of illegitimate rearrangements, suggesting a possible role in preventing rearrangements during the recovery process (16).

In other organisms, RecQ homologs have been shown to have a similar role in preventing strand exchanges and maintaining semiconservative replication. In humans, mutants in BLM fail to maintain semiconservative replication, displaying high rates of sister chromatid exchanges (14, 28). In S. Pombe, Rqh-1 is required to suppress recombination, which leads to an irreversible S-phase arrest (50). In S. cerevisiae, SGS-1 mutants display higher frequencies of chromosome nondisjunction following mitosis (50).

In trying to understand the mechanism by which accurate replication is maintained in the presence of DNA damage, we have further characterized the nascent DNA degradation which occurs prior to the resumption of replication in UV-irradiated *E. coli*. We find that the degradation occurs on the nascent lagging strand of the replication fork and requires functional copies of *recQ* and *recJ*. We present a model to explain how

this processing could function to help RecA filaments stabilize and maintain the strands at the blocked replication fork until the obstructing lesion has been repaired.

Materials and Methods:

Bacterial Strains: SR108 is a *thyA36 deoC2* derivative of W3110 (34). HL946 (SR108 *recF332*::Tn*3*), HL924 (SR108 *recJ*284::Tn*10*), and HL944 (SR108 *recQ1803*::Tn*3*) were made by P1 transduction of the *recF332*::Tn*3*, *recJ284*::Tn*10*, and *recQ1803*::Tn*3* markers from strains HL556, JC12123, and KD2250, respectively, into SR108. HL1034 (SR108 *xonA*::Cat*300*), HL922 (SR108 *recB21 recC22 argA81*::Tn*10*), and HL923 (SR108 *recD1011 argA81*::Tn*10*) were made by P1 transduction of the *xonA*::Cat*300*, *recB21 recC22 argA81*::Tn*10*, and *recD1011 argA81*::Tn*10* markers from strains HR838, V1307, and V220, respectively, into SR108. HL973 (SR108 *recF332*::Tn*3*; *recJ284*::Tn*10*), HL1036 (SR108 *recF332*::Tn*3*; *recD1011 argA81*::Tn*10*), and HL1035 (SR108 *recF332*::Tn*3*; *xonA*::Cat*300*) were made by P1 transduction of the *recJ284*;:Tn*10*, *xonA*::Cat*300*, *recD1011 argA81*::Tn*10*, respectively, into HL 946. The recJ and recQ phenotypes were confirmed by their resistance to thymineless death (39). Three independent isolates of xonA mutants were used in these experiments to minimize the chance of illegitimate P1 transductions. Confirmation of the phenotypes of all other mutants used in this study have been reported previously (6, 7). KD2250 was a gift from H. Nakayama. JC12123 was a gift of A.J. Clark. HR838 was a gift from S. M. Rosenberg.

DNA degradation following UV irradiation: 100μl of a fresh overnight culture was used to inoculate 10 ml of Davis medium supplemented with 0.4% glucose, 0.2% case amino acids, and 10 μg/ml thymine (DGCthy medium) containing 0.2 μCi/ml [^{14}C]thymine. Cells were grown in a 37C shaking water bath to an OD_{600} between 0.3 and 0.4 (approximately 5x10^8 cells/ml). At this time, 1μCi/ml [^{3}H]thymidine was added to the culture. After 10 seconds, the cells were filtered through a 4.5cm, 0.45μ Whatman HA filter. The cells were then washed with 5ml and then 2ml of NET buffer (100mM NaCl, 10mM Tris pH8.0, 10mM EDTA). Washing times varied between 10 and 30 seconds depending on the cell line. The cells were then resuspended in nonradioactive DGCthy medium and irradiated with a dose of 25 J/m2 unless otherwise indicated. The time from placing the cells into nonradioactive media to irradiation was approximately 10 sec. The ^{14}C and ^{3}H remaining in the DNA was measured by averaging duplicate, 0.2ml samples precipitated in 5% cold trichloroacetic acid and filtered onto Whatman glass fiber filters. All zero time points were taken in triplicate rather than duplicate. The initial

values of ^{14}C and ^{3}H were between 900-2000 cpm and 5000-12000 cpm in all experiments.

Degradation of nascent DNA in the leading and lagging strands of the lacZ gene: 0.3ml of a fresh overnight culture was used to inoculate 30 ml of DGCthy medium containing 0.1μCi/ml [^{14}C]thymine. The culture was grown to an OD_{600} between 0.3 and 0.4 before harvesting by filtration and resuspending in DGC medium containing 20μg/ml 5-bromouracil supplemented with 1μCi/ml [^{3}H]5-bromodeoxyuridine (Moravek Biochemical). Within 30 seconds, the culture was filtered again. The cells were then washed with 10ml NET buffer, resuspended in nonradioactive DGCthy medium, and irradiated with 25 J/m2. At the indicated time, 10 ml of cells were placed into an equal volume of ice cold NET buffer, pelleted and lysed in 0.4ml 0.5M K_3PO_4 (pH 11.5). The solution was then subjected to isopycnic alkaline CsCl gradient sedimentation as described previously (48). 30 fractions were collected onto Whatman#17 paper. ^{14}C and ^{3}H in each fraction was determined by scintillation counting. The peak ^{3}H fractions of each time point were slotted in triplicate, twice onto Hybond N+membrane, using a slot blot apparatus. The samples were then probed for either the nascent leading strand DNA (transcribed strand probe) or nascent lagging strand DNA (nontranscribed strand probe) of pZH-10 as described previously (34).

Results:

The nascent DNA degradation requires *recJ* and *recQ*: Nearly 100% of the cells in an exponentially growing culture of *E. coli* survive a 25 J/m^2 dose of short wavelength UV irradiation. Although the ongoing DNA replication is transiently disrupted at this dose, it efficiently resumes following the repair of the UV-induced lesions (7). However, prior to the resumption of DNA synthesis, some degradation of the nascent DNA occurs, as we have previously reported (6, 7). To examine this degradation, cultures grown in [^{14}C]thymine were pulse labeled with [^{3}H]thymidine for 10 seconds, transferred to nonradioactive medium, and irradiated with 25 J/m^2 UV. The amounts of ^{3}H and ^{14}C remaining in the DNA were then followed over time. The ^{14}C prelabel allowed us to compare the degradation occurring in the overall genome to that of the newly synthesized ^{3}H labeled DNA at replication forks. A typical experiment is shown in figure 1. No degradation of the bulk genomic DNA is detected regardless of whether the cells were irradiated or not. No degradation of the nascent DNA is detected in unirradiated cultures after the remaining intracellular pools of [^{3}H]thymidine are rapidly incorporated into the DNA. However in UV-irradiated cultures, the nascent DNA is partially degraded at times prior to the recovery of replication. The increase in ^{3}H

DNA after 60 minutes is probably due to the remaining intracellular pools of [^{3}H]thymidine which are then incorporated when replication resumes.

When the nascent DNA from irradiated *recJ* or *recQ* mutants was examined, no degradation was observed. Figure 2 shows the degradation pattern of mutant strains which were run in parallel to the wild type cells shown in fig 1. The lack of [^{3}H]thymidine incorporation seen immediately following irradiation in *recJ* or *recQ* mutants suggests that the effect is due to a lack of degradation rather than a failure of replication to arrest at sites of DNA damage. That replication is still inhibited by DNA damage in *recJ* and *recQ* was confirmed in experiments in which irradiated and unirradiated *recJ* and *recQ* mutants were compared (data not shown). By contrast to the effect seen with *recJ* and *recQ*, mutants in *xonA* or *recD* , which encode other DNA exonucleases, or *recBC* , which encodes another DNA helicase, did not prevent the nascent DNA degradation from occurring (fig. 2B). Other mutations examined that did not abrogate the degradation of the nascent DNA included *recG*, *mfd*, *uvrA*, and *uvrC* ((7) and data not shown). The amount of degradation in individual experiments typically ranged from 10 to 20% of the nascent DNA in these strains. Whenever less than 20% nascent strand degradation was observed, a correspondingly higher level of reincorporation of [3H] was observed once replication recovered, suggesting that the remaining intracellular pool from the [^{3}H]thymidine pulse was the limiting factor in detecting the degradation. Furthermore, the observed reincorporation following the resumption of replication suggests that the actual amount of degradation detected may be an underestimate of that actually occurring at the replication fork. However, comparisons between mutants from individual experiments, always showed the same differences relative to each other in at least two independent experiments.

Degradation occurs on the nascent lagging strand: Unlike wild type cells, *recF* mutants fail to recover replication following disruption by UV, despite the fact that the removal of UV-induced lesions occurs with kinetics similar to those in wild type cells (7, 42). In the *recF* mutants, the nascent DNA degradation continues beyond the time that replication recovers in wild type cells, but ceases after 50% of a 10 second pulse label has been degraded (fig. 3A) (6, 7). The slight loss of the bulk genomic DNA seen in the *recF* mutants occurs primarily at later times and is thought to be due to secondary effects following the failure of replication to recover normally.

When we examined a *recFrecJ* double mutant, no degradation of the nascent DNA was detected. This suggests that the nascent DNA degradation which occurs in *recF* mutants, although more extensive, is likely to be mechanistically similar to that which normally occurs in wild type cells (fig. 3A).

As was found with wild type cells, mutations in either *recD* or *xonA* also did not alter the pattern of degradation in *recF* mutants (fig. 3B). The degradation of nascent DNA in *recR* mutants, which is similar to that in *recF* mutants, was also found to be dependent upon *recJ* and *recQ*, but was unaffected by mutations in *recD*, *recG*, or *mfd* (data not shown).

We were interested in the observation that although replication does not recover in *recF* mutants, the nascent DNA degradation appears to be limited to approximately 50% of the nascent DNA. Considering the complementing polarities of RecJ and RecQ, we reasoned that the degradation might be limited to one strand of the nascent DNA. To test this idea, we utilized radiolabeled riboprobes corresponding to the leading and lagging strand of replication in the *lacZ* gene. The leading and lagging strand riboprobes were hybridized to DNA which was isolated from irradiated *recF* mutants at various times after UV irradiation. However, because the nascent strand degradation represents such a limited amount of the total genomic DNA, we partially enriched our preparations for the nascent DNA by pulse labeling the *recF* mutants with [^{3}H]5-bromodeoxyuridine. DNA containing bromouracil instead of thymine has a higher buoyant density and can be isolated in isopycnic alkali CsCl gradients. While we could not achieve a complete separation between nascent DNA and bulk genomic DNA due to the short pulse labeling times, we were able to discard a large portion of genomic DNA, which could otherwise interfere with detection of a strand bias in the nascent DNA.

If the nascent DNA on both sides of the replication fork is degraded equally, the ratio between the lagging and leading strands of DNA should remain constant over time (as both strands are degraded). However, if degradation occurs preferentially on one strand, the ratio of the degraded strand relative to the nondegraded strand should decrease over time. When the isolated DNA was probed for the strands of the *lacZ* gene, we found that the lagging strand DNA decreased relative to the leading strand over time, consistent with the degradation occurring preferentially on the lagging strand of the nascent DNA (figure4).

When the analysis was repeated using a *recFrecJ* mutant, that bias in degradation was no longer observed and the ratio between the strands either remained constant or increased, also consistent with the idea that RecJ is degrading the lagging strand of the nascent DNA (figure 4). However, we cannot rule out the possibilities that the degradation within the *lacZ* gene is not representative of the rest of the genome or that the incorporation of 5-bromouracil, which is toxic to E. coli, somehow alters the normal degradation pattern.

Discussion:

We have shown that RecJ and RecQ process the nascent DNA prior to the resumption of replication in UV-irradiated *E. coli*. The observations that the degradation preferentially occurs on the nascent lagging strand in the *lacZ* gene and that only half of the total nascent DNA is susceptible to degradation, support the idea that the nascent lagging strand is partially degraded by RecQ and RecJ in UV-irradiated *E. coli*.

The degradation of the lagging strand is consistent with the known polarities of RecQ and RecJ. The nascent DNA of a disrupted replication fork would be expected to terminate with a 3' end on the leading strand and a 5' end on the lagging strand (figure 5). RecJ, a 5'-3' single strand specific exonuclease, and RecQ, a helicase which translocates in the 3'-5' direction when bound to single stranded DNA, would therefore be expected to displace and degrade the nascent lagging strand. Interestingly, single strand binding protein (SSB), which has been shown to stimulate unwinding of DNA by RecQ, is thought to be present on the single stranded region of the lagging strand template and may help target RecQ in vivo (figure 5) (53). The lack of nascent DNA degradation in either a *recQ* or *recJ* mutant suggests a functional interaction between these gene products in which RecQ is first required to unwind the nascent DNA before it can be degraded by RecJ. The observation that mutations in either *recQ* or *recJ* abrogate the replication fork processing also implies that other nucleases or helicases cannot substitute for this process.

We show that mutations in either *recBC* or *recD* do not prevent the nascent DNA processing. Furthermore, these mutants, unlike *recA* or *recF,* have also been shown to recover replication following UV damage (6, 23). These results are interesting because double strand breaks have been shown to arise at high frequencies in the genomes of some DNA replication mutants (35, 45). Based upon these observations, it has been hypothesized that replication may frequently "collapse" or "disintegrate" upon encountering DNA damage, producing double strand breaks which are then repaired through a recBCD recombination pathway (3, 26, 27, 35, 45). Yet, these observations along with our previous studies imply that, at least for UV-induced DNA damage, replication does not normally recover through a *recBCD* dependent pathway. We would infer that in cells with fully functional replication machinery, double strand breaks may not arise as frequently as have been hypothesized to occur during normal replication. However, exactly why recBC mutants exhibit such poor viability in the presence or absence of DNA damage is an important question which requires further study.

The observation that *xonA* mutants (ExoI) do not prevent the processing is interesting, in that if degradation also occurred on the nascent leading strand, the properties of ExoI would make it a likely candidate. *xonA* encodes a 3'-5' exonuclease

(opposite in polarity to that of *recJ*) that copurifies with both RecA and SSB (1, 2, 29, 36, 40). The lack of effect in *xonA* mutants could suggest that either the leading strand remains protected or that this assay may not relate to the cellular function of *exoI*.

The altered sites and frequencies with which recombination occurs in *recQ* and *recJ* mutants could suggest that the nascent DNA processing helps to suppress chromosomal rearrangements or exchanges when replication is blocked by DNA damage (16, 52). One possibility for how this could occur is that, when replication is blocked, partial degradation of the nascent lagging strand lengthens the triple stranded substrate for RecA protein filaments to polymerize upon at the replication fork (figure 6). By maintaining the pairing of the replication fork strands, RecA may prevent the recombinagenic 3' end of the nascent leading strand from "wandering", potentially pairing at other sites which might share some limited homology.

Most studies on RecA-mediated homologous strand pairing have focused primarily upon its role in catalyzing recombination events. However as discussed in the introduction, during genomic replication, the presence of RecA is absolutely required to maintain replication forks when they become blocked by DNA damage (21, 22). Purified RecA binds single stranded DNA and progressively pairs it with homologous duplex DNA in a 5'-3' direction to form a triple stranded structure (for reviews see (25, 41, 49)). During recombination, this activity is thought to be critical in bringing together homologous pieces of DNA. During the processive, semiconservative replication of the genome however, this same activity could also be expected to play a critical role. To understand how the biochemical activity of RecA may operate to stabilize replication forks, consider that the template of an ongoing replication fork contains one strand which has not yet been replicated (single stranded DNA) and one strand which has been replicated (duplex DNA that is homologous to the single stranded region) as shown in figure 5. Given these substrates, RecA would be expected to maintain a joint molecule between the replicated template strand and the nonreplicated template. The rapid degradation of the genomic DNA which occurs when replication encounters a DNA lesion in *recA* mutants suggests that this pairing is required to protect the replication fork from degradation. Importantly however, maintaining this pairing would also preserve the accurate and semiconservative duplication of the template once replication could again resume (i.e. after the blocking lesion has been repaired).

The role of RecJ and RecQ in processing disrupted replication forks is provocative when one considers their function in assays which score for recombinational events. To catalyze recombinational events, RecJ and RecQ are thought to function by creating single stranded DNA extensions for RecA-mediated homologous strand pairing

(19, 25). During genomic replication, the DNA substrate processed by RecJ and RecQ is at the blocked replication fork, suggesting that by creating single stranded DNA at this site, they promote homologous pairing between the strands of the replication. In a sense, no substrate could be a more legitimate site for homologous strand pairing than the original site of blockage! This is the only site in the cell through which homologous strand pairing would maintain the semiconservative replication of the genome.

RecQ belongs to a highly conserved family of helicases which has been shown to be important for maintaining genomic integrity and semiconservative DNA replication. The genes defective in the rare genetic disorders of Bloom and Werner syndromes encode DNA helicases which share large portions of homology with the *E. coli* RecQ protein (8, 56). At the cellular level, these disorders are associated with high rates of sister chromatid exchanges, chromosomal rearrangements, and a general genomic instability (10, 28, 37). Bloom syndrome cells are hypersensitive to UV and exhibit abnormal replication patterns following DNA damage (15, 30). Cells from Werner syndrome patients display prolonged or abnormal replication during S-phase (17). Patients with Bloom syndrome exhibit growth retardation, decreased fertility, immune deficiencies, and an increased incidence of cancer (14). Werner syndrome patients also exhibit growth retardation, decreased fertility, and cancer predisposition but additionally exhibit characteristics of accelerated or premature aging (9).

RecQ homologs in other organisms also have phenotypes associated with maintaining semiconservative DNA replication. In *S. Pombe*, Rqh-1 is required to suppress recombination which leads to irreversible S-phase arrest (50). In *S. cerevisiae*, mutations in SGS-1, a RecQ homolog that interacts with topoisomerases II and III, are associated with increased rates of chromosome nondisjunction (13, 54, 55).

At the level of the chromosome, the frequency of strand exchange during genomic replication correlates directly with cell death, genomic instability, and in higher organisms, a predisposition to cancer. This observation implies that there is a major conceptual difference between a protein which is required for recombination to occur and a recombination protein. Many proteins have been isolated because they affect recombination frequencies. Quite naturally, these proteins have been characterized primarily for their ability to rearrange DNA. During an asexual reproductive cycle however, many of these proteins, including the *E. coli* RecA protein, are intimately associated with the ability of cells to carry out the replication of the genome. The fact that genomic replication is semiconservative suggests that these proteins may help maintain this process. Recombination events may often represent the products of tolerated, but inappropriate, resolutions to strand pairing events. Understanding the

circumstances which can lead to these strand exchanges and rearrangements however, is critical to understanding how genomic stability is maintained. Both RecJ and RecQ were identified as proteins which were required for recombination to occur under certain conditions (31, 38). However on the chromosome, RecJ and RecQ appear to function at the replication fork. That the cellular target of RecJ and RecQ in *E. coli* is the nascent DNA of blocked replication forks suggests that their role in maintaining genomic stability could be linked to their ability to maintain the replication fork during *rec*overy rather than *rec*ombination.

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Figure Legends:

Figure 1. Degradation of the nascent DNA following UV irradiation. [^{3}H]Thymidine was added to [^{14}C]thymine prelabeled cells 10 seconds before the cells were placed in nonradiolabeled medium and either UV irradiated with 25 J/m^{2} or left unirradiated. The relative amount of radioactivity remaining in the DNA is plotted over time. ^{14}C-genomic DNA (open symbols); ^{3}H-nascent DNA at the growing fork (filled symbols, labeled on graph). (GB) Irradiated cells; (AF) Unirradiated cells.

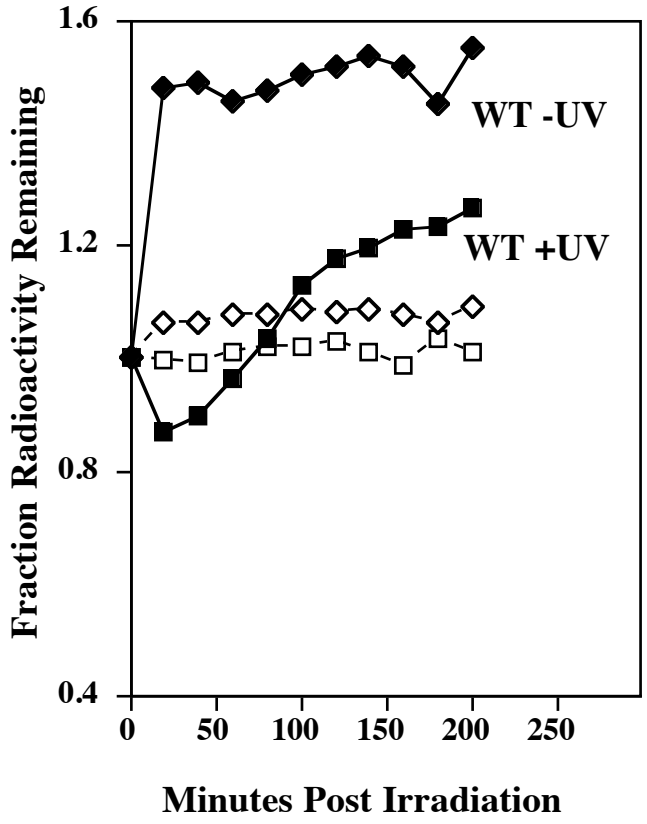


Figure 2. Degradation of the nascent DNA does not occur in *recQ* or *recJ* mutants. [^{3}H]Thymidine was added to [^{14}C]thymine prelabeled cells 10 seconds before the cells were placed in nonradiolabeled medium and irradiated with 25J/m^{2}. The relative amount of radioactivity remaining in the DNA is plotted over time. ^{14}C-genomic DNA (open symbols); ^{3}H-nascent DNA at the growing fork (filled symbols, labeled on graph). A.) (GB) Parental cells; (CH) *recJ* (SP); *recQ* B.) (GB) Parental cells; (Q) *recBC*; (R) *recD*; (ÅF) *xonA*.

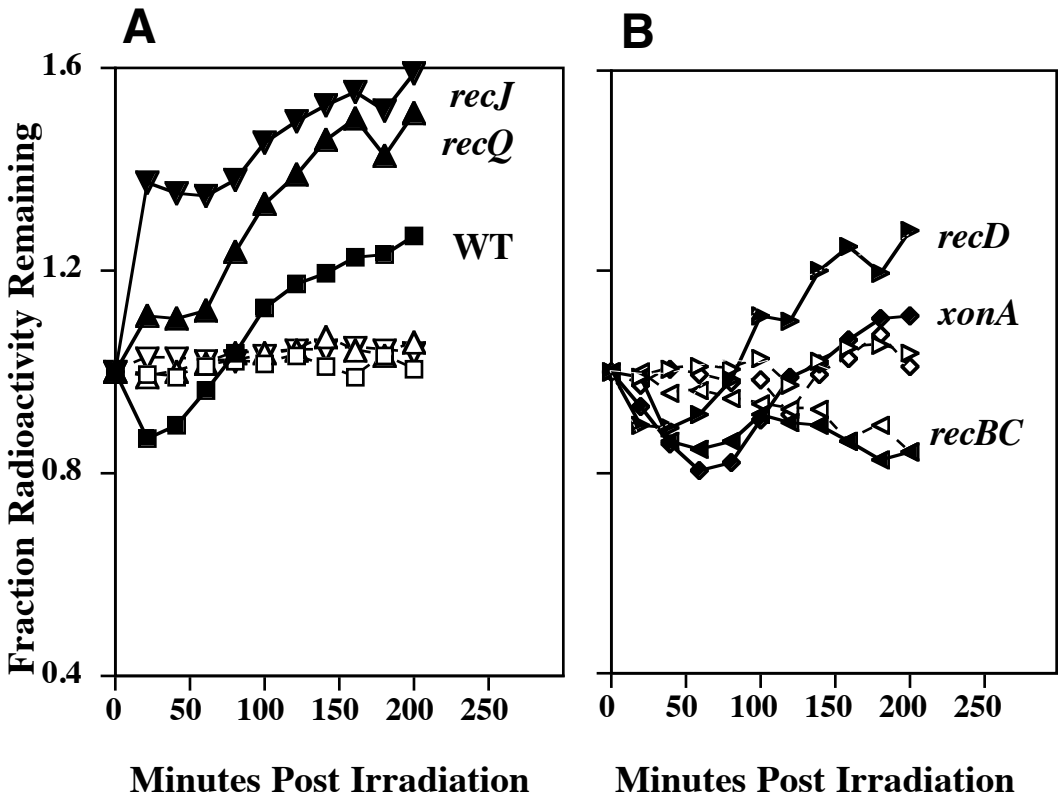


Figure 3. The extensive degradation of nascent DNA in *recF* mutants does not occur in the absence of *recJ*. [^{3}H]Thymidine was added to [^{14}C]thymine prelabeled cells 10 seconds before the cells were placed in nonradiolabeled medium and irradiated with 25J/m^{2}. The relative amount of radioactivity remaining in the DNA is plotted over time. ^{14}C-genomic DNA (open symbols); ^{3}H-nascent DNA at the growing fork (filled symbols, labeled on graph). A.) (GB) *recF*; (ÉJ)*recFrecJ*. B.) (GB) *recF*; (CH) *recFrecD*; (ÜP) *recFxonA*.

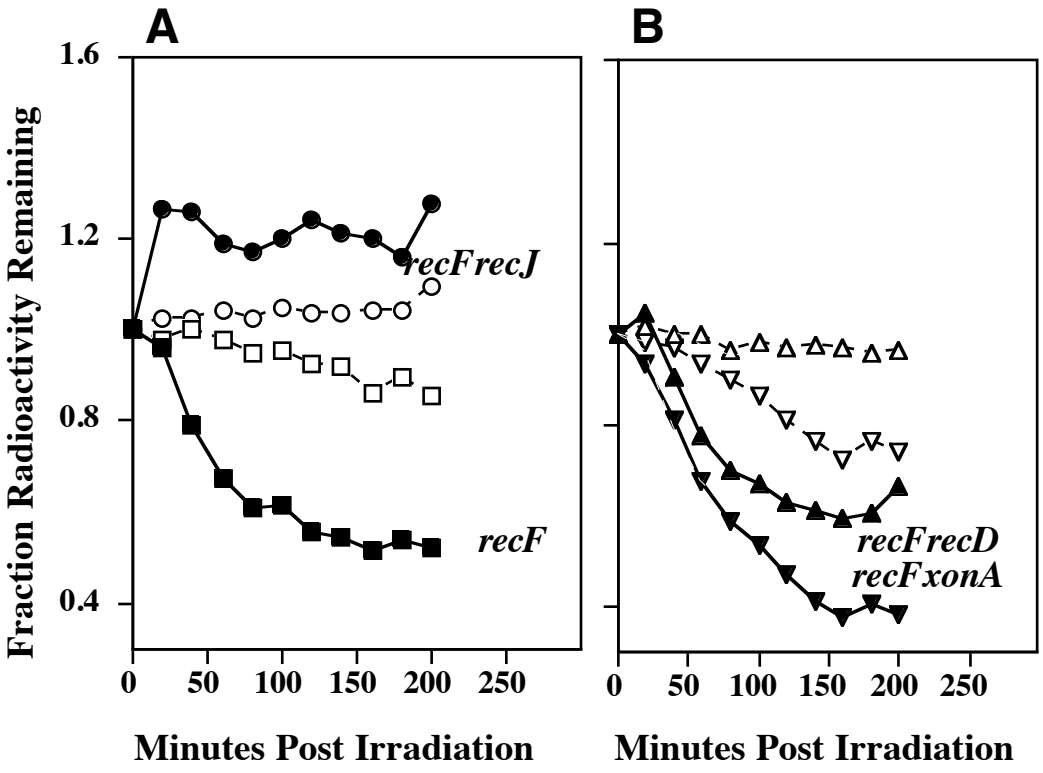


Figure 4. The nascent lagging strand of the *lacZ* gene is preferentially degraded following UV irradiation in *recF* mutants but not *recFrecJ* mutants. DNA enriched for the nascent DNA was isolated at various times following 25 J/m^2 of UV irradiation. The isolated DNA from each time point was hybridized with riboprobes corresponding to either the leading or lagging strand of the *lacZ* gene and the amount of hybridization in each case was quantitated. The ratio of the lagging strand signal to the leading strand signal at each time point is plotted. (BGG) *recF* from three independent experiments; (ÉJ) *recFrecJ* from two independent experiments.

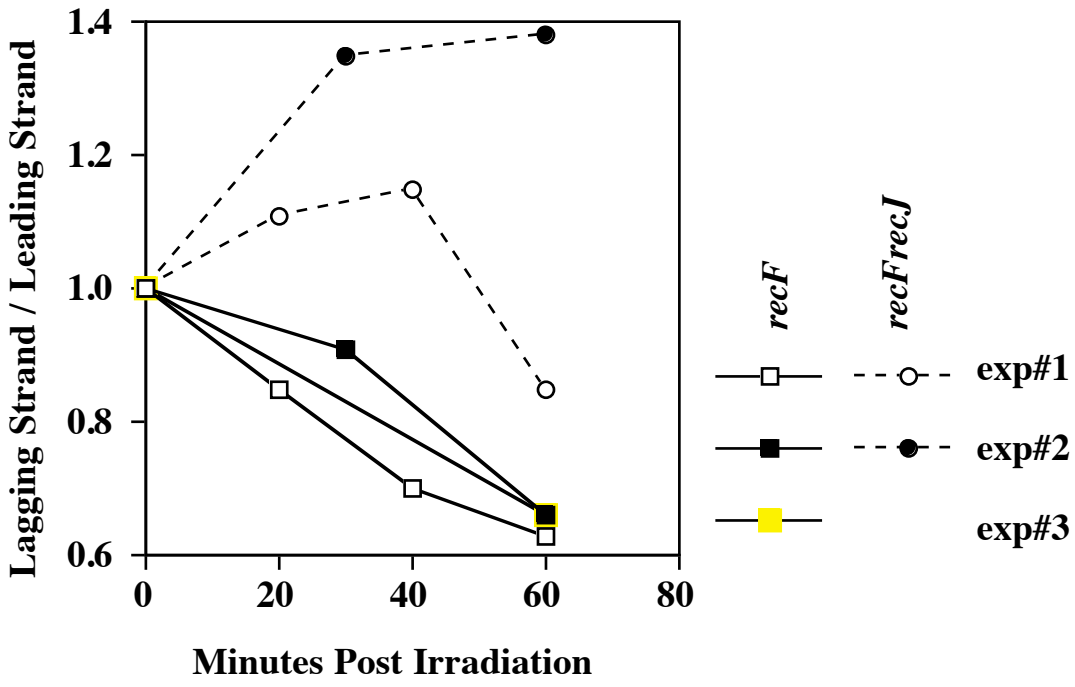


Figure5. Proposed Degradation of the Nascent DNA by RecJ and RecQ. During semiconservative replication, concurrent DNA synthesis in a 5'-3' direction on both strands of the DNA template creates a single stranded region on the lagging strand template. The single stranded region is thought to be bound by (SSB) and will vary in length depending upon the placement of the previous lagging strand primer. Following the premature disruption of replication, the nascent DNA is expected to terminate with a 3'end on the leading strand and a 5'end on the lagging strand, as shown (i). RecQ, upon binding to the single stranded region, translocates in a 5'-3' direction, displacing the nascent lagging strand and making it susceptible to degradation by RecJ, a 5' single stranded exonuclease (ii). The processing creates an extended single stranded region on the lagging strand template at the site of the disrupted replication fork (iii).

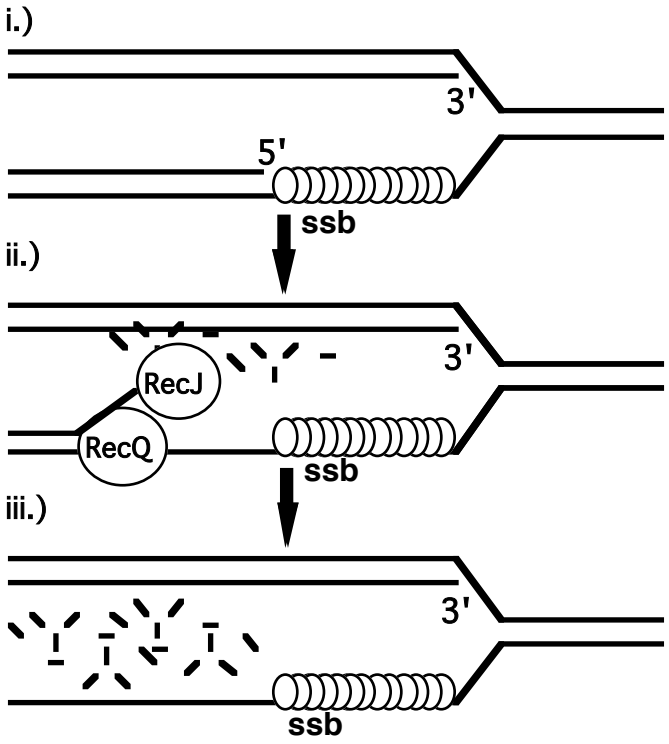
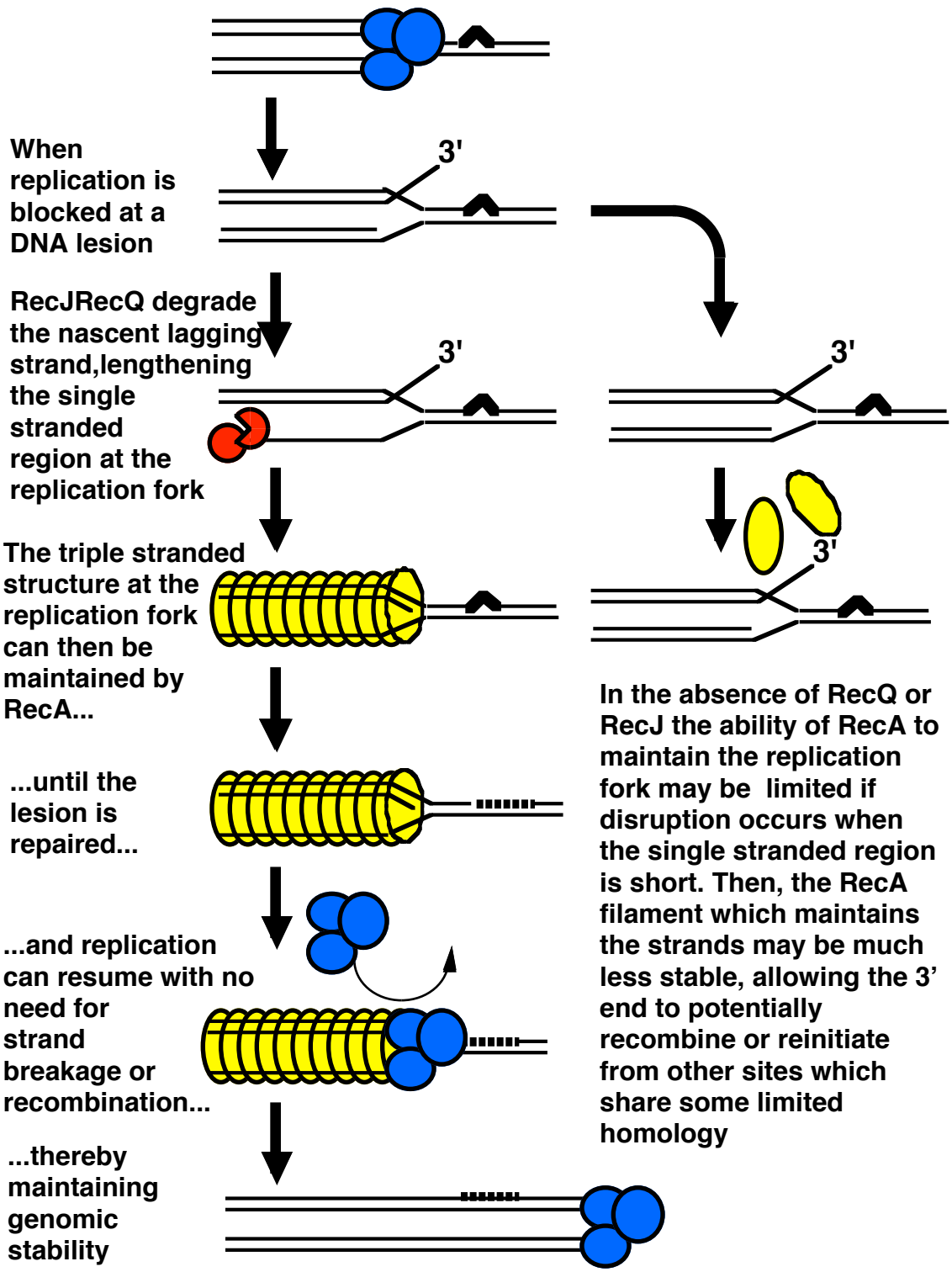


Figure 6. Model for suppression of recombination by RecQ and RecJ.



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