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Recombination-dependent DNA replication in bacteriophage T4: An evolutionary study

McCreary, Ronald Patrick, Ph.D.

The University of Arizona, 1990

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RECOMBINATION DEPENDENT DNA REPLICATION IN BACTERIOPHAGE T4: AN EVOLUTIONARY STUDY

by

Ronald Patrick McCreary

A Dissertation Submitted to the Faculty of the

COMMITTEE ON GENETICS (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Ronald Patrick McCreary

entitled Recombination Dependent DNA Replication in Bacteriophage T4: An Evolutionary Study

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Dr. Dennis Ray	10/29/90 Date
Dr. Junetsu Ito	10/29/90 Date
Dr. Richard Freidman	10/29/90 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Dr. Harris Bernstein	10/29/90 Date

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SIGNED: R. Patrick McCann Jr.

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ABSTRACT

Genetic recombination is an ubiquitous process in living organisms and is one of the most elaborate activities that DNA undergoes. As such, the recombination process potentially interacts with all of the major metabolic events involving DNA. This study examined one of these interactions, specifically that occuring between recombination and DNA replication in phage T4. This interaction was investigated from both the mechanistic and evolutionary viewpoints.

The central reactions of homologous recombination, homologous pairing and strand insertion, are generally catalyzed by a single enzyme. The E. coli recA gene product serves as the paradigm for this class of enzyme. Four cloned wild type 'recA' genes from the bacterial species E. coli, A. caviae, and B. pertussis and the uvsX gene from phage T4, were used to test for complementation of both E. coli recA$^-$ and phage T4 uvsX$^-$ mutants. The B. pertussis recA$^+$ gene was able to complement an E. coli recA$^-$ mutant with respect to the repair of UV induced DNA damage and general recombination. In a phage Mode 2 DNA synthesis assay, all three bacterial recA genes complemented a uvsX$^-$ mutant to some extent, but the B. pertussis RecA protein allowed 'runaway' DNA synthesis to occur. In contrast, none of the three bacterial genes were able to increase DNA synthesis of a uvsX$^+$, uvsY$^-$ phage T4. In a one step growth experiment, only the B. pertussis RecA protein complemented a phage uvsX$^-$ mutant. However, none of the three bacterial recA genes

complemented the same phage mutant in a UV survival assay. These results imply that normal wild type Mode 2 phage DNA synthesis is an example of a specific interaction between the DNA replication and homologous recombination 'machines' of phage T4.

Multiplicity reactivation (MR) is a type of recombinational repair. MR experiments were performed using a gene 42(ts) mutant phage to study the effect of this member of the phage's DNA replication machinery on recombinational DNA repair. The data show that MR is dependent on the gene 42 product.

A hypothesis concerning the molecular coevolution of DNA replication and recombination pathways is developed. This hypothesis is used to explain the significantly different ways in which each of the enzymatically equivalent 'recA' proteins substitutes for the E. coli RecA protein and gpuvsX.

Chapter I

INTRODUCTION

The term "genetic recombination" has acquired a plethora of meanings in modern genetic literature for a myriad of reasons. These reasons include the ubiquity of the event in living organisms, the multitude of cellular pathways in which it functions, and the multiple "levels" at which the phenomenon manifests itself. In sexually reproducing organisms, most of the recombination between unlinked genes occurs as a result of the independent assortment of non-homologous chromosomes (92, 116, 139). However, in keeping with the definition of Bridges and Morgan, I will use the term genetic recombination to refer to the process of changing the linear relationship between genes on homologous chromosomes, that is as "...a shortening of the full term 'recombination of linked characters.'" (14). It is useful to define recombination in this way when addressing questions about the mechanisms of genetic exchange between homologous chromosomes. Thus we can refer to this process as the "mechanism of recombination". One approach to this problem is to analyze the progeny of a mating for new linkage patterns of genes. This approach can give insights about the recombination events that gave rise to these new patterns.

The study of recombinant progeny formed the initial knowledge base from which much of our understanding of the mechanism of

recombination has been obtained. These studies provided insights into the nature of gene organization as well. For example, Sturtevant (138) using his own data from six factor "crossover experiments" in Drosophila, plus recombination data of several others, was able to definitively show that genes are arranged in a linear order along the chromosomes. The method he developed proved to be widely applicable to other organisms. It enabled geneticists to "map" genes in a linear array based on the amount of recombination between them (14). Sturtevant's work also gave insights into the nature of genetic recombination. From his study of unequal crossing over at the Bar locus (137), he gained understanding of the key events occurring during genetic exchange; homologous pairing in register (i.e. side by side) followed by physical exchange of genetic information. Genetic recombination events were shown to correlate with the cytologic events of meiosis by the definitive work of Creighton and McClintock (29). In this study the authors demonstrated a one-to-one correlation between the genetic event of recombination and the cytologic event of physical exchange of the corresponding portions of two homologous chromosomes. Further insights into how much recombination was possible for a given karyotype and how recombination might be controlled were presented by Darlington (31). He introduced the concept of Recombination Index (derived by summing the haploid number of chromosomes and the average number of chiasmata per nucleus) to arrive at a numerical value for recombination both between homologs and non-homologs that would be characteristic for

a particular karyotype. Inherent in this idea was the notion that the number of recombination events per genome is regulated by the cell.

After it was established that DNA is the principal genetic material (5, 49) and its structure was elucidated by Watson and Crick (147), further progress was made toward understanding the nature of the gene and the recombination event itself by combining the "classical" recombinant studies with the "molecular" evidence. In 1955 Seymour Benzer (7) applied this approach to dissect the rII region of bacteriophage T4 into its two component genes using a "cis-trans" complementation assay. With this experimental technique, Benzer was the first to argue convincingly that the recombination event occurred between individual nucleotide pairs and that it apparently could happen anywhere along the chromosome. Efforts to understand the mechanism of recombination now focused on finding a workable model to explain the observed recombination data in terms of the structure of DNA.

Models of Recombination

The first widely accepted molecular model of genetic recombination was proposed by Robin Holliday in 1964 (54). Holliday offered an alternative to the then prevalent "copy choice" models for explaining aberrant segregation in fungi. Aberrant segregation involved unequal recovery of genetic markers following genetic recombination. This phenominon was first called gene

conversion by Winkler, underscoring the apparent conversion of one allele into another that is the hallmark of this recombination event (116). The copy choice models of genetic recombination were based on the idea that DNA replication (the "S" phase of the meiotic cell cycle) occurred during or after chromosome pairing. With chromosome pairing and DNA replication proceeding in that order, or at least as simultaneous events, the copy choice models proposed that the replication machinery could switch templates over a short region of homologous pairing, and that this switching need not be reciprocal with respect to any given locus. Thus "copy choice" could explain at least some types of gene conversion events as well as "normal" genetic crossing over in which there was no aberrant segregation. One of the main difficulties with copy choice models was that they were not consistent with the semiconservative nature of DNA replication. To avoid this problem, as well as others raised by the copy choice mechanism, Holliday proposed a model for the origin of gene conversion events based on genetic recombination where the breakage and reunion of the DNA strands between chromatids was the driving molecular event, not DNA replication.

Figure 1 depicts the meiotic recombination event as proposed by Holliday. For clarity, I have shown in Figure 1, as well as in Figures 2 and 3, only the the two duplex DNA molecules that comprise the two chromatids actually involved in the recombination event even though meiotic recombination occurs at the four chromatid stage. The central event in Holliday's model involved

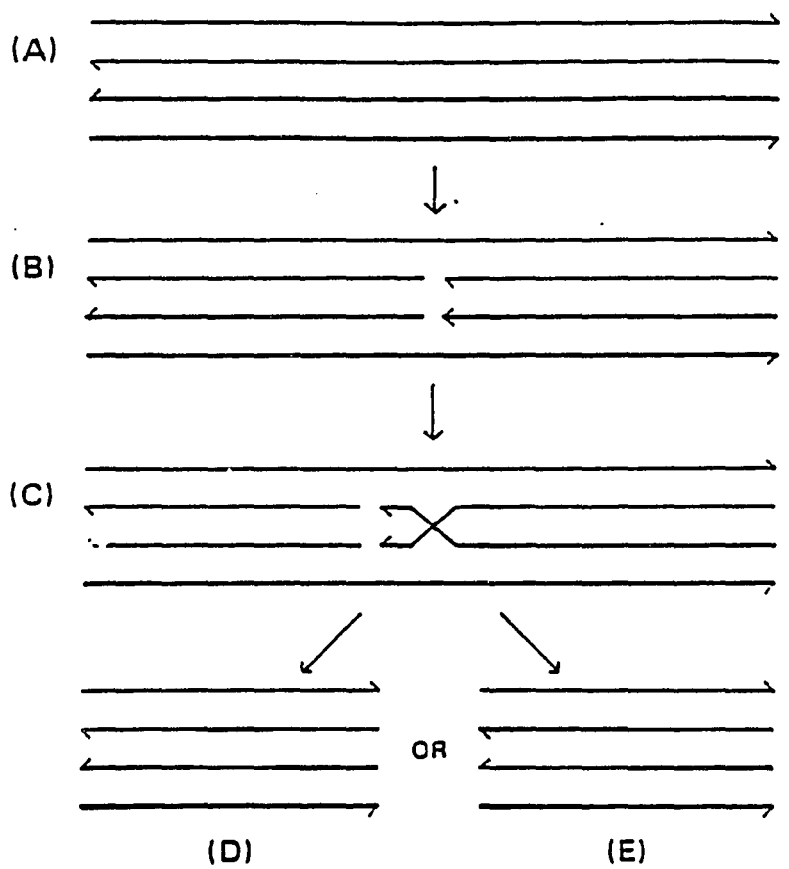

Figure 1. The Holliday Model. Two homologous DNA molecules each composed of two strands of opposite polarity are indicated. Two strands of the same polarity in different DNA molecules are nicked at homologous sites (B). The free ends of these two strands can be exchanged to form a cruciform intermediate referred to as a "Holliday junction" (C). This Holliday junction can be resolved in favor of either non-recombination of flanking loci (D) or recombination of flanking loci(E).

the breakage of one of the strands of the double helix in each paired homologous chromatid and the physical switching of these strands. The resulting cruciform, or chi, structure predicted by Holliday in his model has been theoretically and experimentally verified (34, 94, 109) and is now known as the Holliday junction or Holliday intermediate. Two outcomes are possible from the resolution of the Holliday junction owing to its ability to isomerize (34, 94). If the two originally crossed strands are cut, then the resulting chromatids will not be recombinants with respect to flanking loci. However, they will have a "short patch" of heteroduplex DNA. If, however, the two noncrossed strands are cut, then genetic recombination for flanking loci is the result. The Holliday intermediate has served as a unifying concept in the molecular study of recombination, providing a link between the genetic events of recombination and the structure of DNA. The Holliday model incorporated two other conceptual features that have been carried forward in subsequent models of recombination. These are: inclusion of DNA repair as part of the recombination process, and strand breakage as essential for the initiation of recombination. In the Holliday model, regions of symmetric heteroduplex DNA are formed as a result of strand exchange between heteroallelic loci. Holliday, as well as subsequent investigators, left the repair of mismatches (mispaired bases) that might arise in the heteroduplex region to "...enzymes which can repair points of damage in DNA..." (54). Gene conversion then, was the result of the "repair", or correction, of these regions of mismatched bases.

A mismatch can be "corrected" in either of two ways, depending on which base of the mismatched pair is retained and which removed. Thus the outcome of a mispair correction event depends on which strand is retained as the template. This merging of two metabolic pathways, "breaking-rejoining" and mismatch repair, to explain genetic recombination has proved consistent with a great deal of experimental evidence on the nature of recombination and foreshadowed the necessity of considering the process in a larger context than a simple singular enzymatic event.

As research continued into the nature of the recombination process, the validity of the Holliday model with respect to heteroduplex DNA formation via the predicted intermediate became more certain. However, data from several investigations of fungal recombination began to show a pattern of gene conversion that could not be explained by the initiation sequence proposed by Holliday. Specifically, work by Stadler and Towe on Ascobolus indicated that heteroduplex DNA is often formed in only one of the homologous chromosomes involved in meiotic recombination rather than both (129). Furthermore, work by Rossignol et al. (117), and Hurst et al. (57) demonstrated that the heteroduplex DNA was often not formed symmetrically when two homologous chromosomes recombined. This asymmetry ran counter to the predictions drawn from the simultaneous single strand breaks and reciprocal exchange of strands proposed by Holliday as the initiation event in recombination (Figure 1). At this point much of the research effort on the mechanism of recombination began to focus on the

initiation of recombination, where it remains to this day.

In 1975, Meselson and Radding proposed a model that had as its centerpiece a modification of the process leading to the formation of a Holliday junction (95). They suggested that the initiation event was a two step process that began with the "nicking" of only one of the paired duplexes (Figure 2). They then introduced the concept that this nicking event was coupled to DNA synthesis. DNA synthesis progressing from one end (the 3' -OH end) of the nicked strand would generate a single strand section of DNA that would be free to invade the paired duplex at a region of homology (step C, Figure 2). This would form a "D loop" structure (see step D, Figure 2). The D loop could then be expanded into a region of heteroduplex DNA (step D, Figure 2). The "D loop" would be cut and the resulting single-strand of DNA could then complete the reciprocal exchange resulting in the formation of the Holliday junction but at a slightly displaced location from the original nicking event. In this manner heteroduplex DNA arises in both chromatids, but the two regions are asymetrical. Two asymetrical heteroduplex regions are produced as an antecedent to each recombination event. The Meselson-Radding model thus supplied an explanation for the fungal data, but in doing so, added a degree of complexity beyond that of the Holliday model. By coupling DNA synthesis to recombination, Meselson and Radding introduced another molecular process to the overall recombination mechanism. This addition brought several more enzymatic steps into play and also implied an increased degree of regulation to coordinate the three

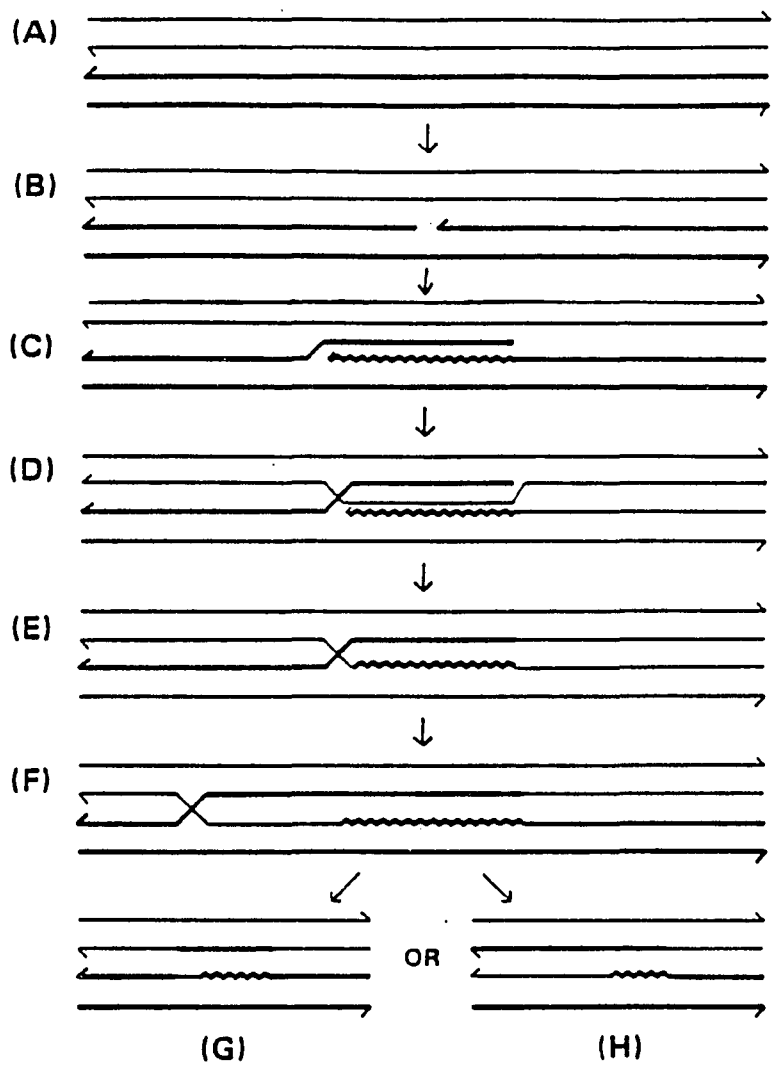

Figure 2. The Meselson-Radding Model. A single strand of one of the two homologous chromatids is nicked (B) to yield a free 3' end which can act as a primer for DNA synthesis (C). This synthesis results in a displaced strand free to invade the other duplex forming a "D-loop" (C and D). Branch migration results in a region of asymmetrical heteroduplex DNA (E and F). Resolution of the Holliday junction yields progeny that are either non-recombinant (G), or recombinant (H) with respect to flanking loci.

processes of (1) resolving the Holliday junction, (2) mispair correction and (3) DNA synthesis.

The Meselson-Radding model of recombination met with approval due to its greater precision in explaining the genetic data of fungal recombination, especially the data implying the existence of asymmetric heteroduplex DNA. The model also incorporated evidence on the physical structure and mechanical properties of the DNA in recombination intermediates. Biochemical evidence supporting the existence of the D-loop structure predicted in the Meselson-Radding model has come, from studies on the recA protein of Escherichia coli (6, 123, 124). As will be discussed below, the recA protein is able to catalyze several key enzymatic steps central to both models of recombination discussed so far. These steps include homologous pairing and single strand insertion into a homologous duplex which leads directly to the formation of the predicted D-loop structure. However, as more genetic data concerning the meiotic events in fungi were obtained, this model of recombination became increasingly encumbered with modifications and assumptions. For example, Fogel et al. (36) examined the frequency of aberrant segregation at the arg4 locus in yeast and found that there appeared to be a gradient of conversion events from one end of the locus to the other, reconfirming the concept of polarity of gene conversion events. To account for this, special assumptions concerning the nature of gene conversion at the "initiators" or "terminators" had to be invoked (84, 130, 140). Another concern developed with regard to the information flow between the two

interacting strands, that is, whether the homolog initiating the recombination process was the recipient or the donor of information to the other homolog. The Meselson-Radding model favored information flow from the "initiator" while subsequent data indicated that the initiator homolog was the recipient of information (84). While these concerns did not disprove the Meselson-Radding model, they led investigators of recombination to begin to reexamine the recombination process, especially with respect to potential initiation mechanisms.

In 1983, a significantly new approach to the question of how recombination is initiated was proposed by Szostak et al. (140). The double-strand break repair model which they proposed, brought yet another level of complexity to the recombination model. The model attempts to provide a unified explanation of the data on recombination from not only eukaryotes (primarily yeast and other fungi), but also from phage and bacteria. This model is outlined in Figure 3. The central feature of the model is, as its name implies, the assumption that a double-strand break is the first event in the strand exchange. The initiating double-strand break is made in the "recipient" chromatid. This break is then enzymatically widened into a gap. Next, a 3' end from the recipient strand invades the "donor" strand to form a D-loop. This D-loop is expanded by a round of DNA synthesis, using the free 3' end as the primer, in a manner similar to that proposed by Meselson and Radding. As the D-loop is widened the displaced "donor" strand is free to accomplish a reciprocal "strand invasion" into the

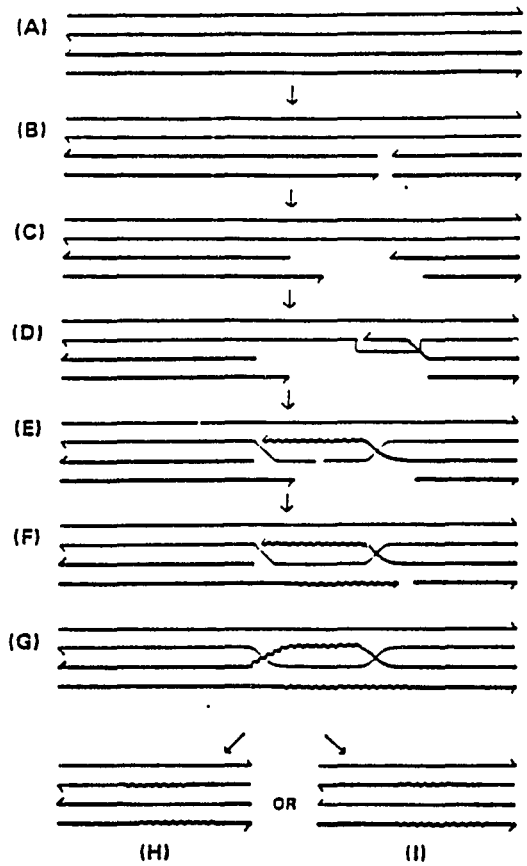

Figure 3. The "Double-Strand Break" Repair Model. Two duplex DNA molecules are shown each with two antiparallel strands. A double strand break is formed in the "recipient" DNA which is then widened into a gap (B and C). Strand invasion occurs into the "donor" duplex by one of the free 3' ends and a round of DNA synthesis is initiated. Then a Holliday junction is formed (D and E). A second round of DNA synthesis using the displaced donor strand as template occurs resulting in the formation of a second Holliday junction (F and G). Resolution of the two Holliday junctions in like manner results in products that are non-recombinant for flanking regions (H). When the two Holliday junctions are resolved in a dissimilar fashion, the flanking loci are recombinant (I).

double strand gap where the remaining free 3' end from the recipient can then serve as a primer for a second round of repair, in this case "gap filling", DNA synthesis. Thus, two Holliday structures are formed, with associated regions of asymmetric heteroduplex DNA, as a result of each "strand invasion" event. The manner in which each of these Holliday structures is resolved is the determining factor in whether the resulting progeny chromosomes would be nonrecombinant for flanking regions (both Holliday structures resolved in the same manner) or recombinant for flanking regions (each Holliday structure resolved in the opposite fashion).

The recombination model proposed by Szostak et al. was not the first to highlight a potential role for double-strand breaks in recombination (115). However, it does have several unique aspects. First, the primary source of new experimental data did not come strictly from the analysis of yeast or other fungal meiotic products but from the results of plasmid transformation experiments (51, 106, 107). This broadening of the experimental foundation has helped in making this a more unifying model of recombination than its predecessors. Another new idea proposed in this model concerns the role of heteroduplex DNA in gene conversion. The double-strand break model proposes two pathways for the gene conversion event. These alternatives depend on where the genetic markers used to detect gene conversion are located (see Figure 3). If the genetic marker is in one of the two regions of heteroduplex DNA, mismatch correction can be the mechanism by which gene conversion is achieved as in the previously described models. If, however, the

genetic marker is in the area deleted by the double strand gap, then the gene conversion event does not occur through a heteroduplex intermediate but by direct transfer of information from the donor chromatid.

Finally, the double-strand break repair model of recombination, by postulating the formation and resolution of two Holliday structures, two rounds of mismatch correction, and two rounds of DNA synthesis has a degree of complexity and implied regulation, well beyond any of its predecessors. This sophistication may be a reflection of the high stake the cell has in ensuring that once initiated, the recombination process be brought to successful fruition. Failure to successfully complete a recombination event may result in the loss of genetic information and possibly in the death of a progeny. If the double-strand break repair model is indeed closer to the actual mechanism than its predecessors, then it strongly implies an intertwining of genetic recombination with replication and repair beyond what was previously supposed.

The development of the double-strand break repair model has, as with the other models, focused on explaining both the genetic and biochemical evidence. However, unlike its predecessors, the model is intended to explain evidence from a wide range of experimental systems. While the preponderance of the data, especially the genetic data, has fit the double strand break repair model rather well, a truly differentiating experiment between the double-strand break repair model and versions of the Meselson-

Radding model has yet to be done. The strongest body of evidence supporting the double-strand break repair model concerns the role of a double-strand break in recombination. In bacteriophage lambda, work exploring the phage's homologous recombination system (the so called Red system) strongly implicates double-strand breakage at the cos site as being the initiating event in the phage's recombination pathway (119, 131-133, 141). Likewise, the recBCD recombination pathway in Escherichia coli seems to be primarily initiated by double-strand breaks at Chi sites (18, 19, 127, 142). In yeast, double-strand breaks have been shown to be involved in at least one pathway of initiation of meiotic recombination (70), and in both mitotic and meiotic gene conversion events (20, 105, 118) as well as in gene conversion during mating type switching (87, 136). In mammalian systems the double-strand break has been related to the process of homologous recombination as well (13, 128). Thus the position of the double-strand break as a significant participant in a whole range of recombination pathways from phage to mammals appears to be well established.

As double-strand breaks or gaps continue to be identified as early events in various recombination pathways, the exact nature of their involvement remains unclear. The uncertainty arises from the equivocal interpretation of the data. For example, the data presented by Fogel et al. and Hastings (36, 47) concerning the degree of association of gene conversion with crossing over of flanking regions was cited by Thaler and Stahl (143) in their argument for the necessity to modify the double strand break model

so that it more resembled the one offered by Resnick (115). However, Borts and Haber (11) argue that the observed differences in gene conversion tract lengths that promoted concern from Thaler and Stahl, are simply due to differences in experimental methodology. In a careful study of mitotic and meiotic gene conversion events involving insertion mutations, Vincent and Petes (144) were able to explain their findings equally as well using either the Meselson-Radding or Szostak et al. models. In addition to the ambiguity arising from the genetic data, there is uncertainty in the biochemical data as well. On the one hand, the enzymology of the recBCD and recA systems are relatively well understood (20, 142, 146) (see below). On the other hand, the double Holliday junction intermediate proposed in the Szostak et al. model has only been verified in vitro (64, 65), but evidence for its existence in vivo has not been forthcoming despite an extensive search (143).

The difficulty distinguishing experimentally between the various models of recombination, those discussed above and several others that have been put forth, can be attributed to several causes. First, the complexity of the recombination process itself, coupled with our incomplete understanding of the major interacting pathways such as replication and repair, is a barrier to designing critical experiments. This is especially true with respect to eukaryotic systems. Second, recombination manifests itself, and is studied, in such a myriad of ways at the cellular and molecular level (e.g. meiotic and mitotic recombination, viral and bacterial

recombination, to name just a few), that integration of all the data available is difficult. Thirdly, as observed by Thaler and Stahl (143), the tendency of biologists to keep searching for a single unifying model which encompases each and every recombination system may be obscuring the true diversity of the process.

The recA Gene Function

Mechanistically, genetic recombination is generally subdivided into the two catagories of (i) general recombination between DNA homologs and (ii) site specific recombination (28). The distinction between these broad classes appears to be straightforward. Homologous recombination is that recombination which occurs, more or less randomly, between two DNA molecules with substantial sequence homology. Site specific recombination refers to cases where recombination can occur only at very specific DNA sequences (73). Although the genetic consequences of these two catagories of recombination are quite distinct (43), if one continues the comparisons down to the molecular level the similarities between these genetically distinct events become apparent. For example, in conservative site specific recombination such as that catylized by the Int protein of phage Lambda and the FLP protein encoded by the 2-micron plasmid found in S. cerevisiae (28, 43, 56), a Holliday intermediate, apparently identical with that described above in the models for homologous recombination, has been identified and characterized (53, 56, 62, 63, 75, 104).

This overlap in intermediate structures can be most easily explained by the fact that, at the DNA level, there are limited ways in which the DNA strands can be manipulated. Therefore each of these general types of recombination pathways have evolved the same, or apparently the same, "mechanical" solution with respect to DNA manipulations, but remain distinctly different when observed at the genetic or enzymatic levels.

Homologous and site specific recombination proceed via independent, and for the most part non-interchangeable, enzymatic (genetic) pathways (28). Here, we will focus on homologous recombination. At the heart of homologous recombination is the homologous pairing-strand insertion step leading to the formation of first the "D-loop" and ultimately, to the Holliday intermediate. This entire set of reactions, in homologous recombination in E. coli, is catalyzed in by a single protein--the recA gene product (110). The recA gene was first identified by Clark and Margulies in 1965 using several E. coli mutants that had dramatically reduced levels of genetic recombination (22). In the intervening years, extensive genetic and biochemical analyses of the recA gene and the RecA protein have elucidated the molecular mechanism of homologous recombination. These studies have shown that the recA gene product, a monomer with a molecular weight of about 38,000 daltons (111, 126), is a bifunctional protein. It is a rather exquisite example of economy of biologic function. The recA protein serves as both a recombinase, catalyzing the central reactions of homologous recombination, and as a key regulatory element in the so

called SOS regulon of E. coli through its "protease" function (81, 110, 111, 145). The SOS regulon is a set of genes under the control of the recA gene product. These genes, when induced, provide a response to DNA damage which includes those genes necessary for DNA repair. With respect to its regulatory function, the recA "protease" becomes activated in the presence of DNA damage or blocked replication (79, 80, 125). Once activated, the "protease" mediates the autodigestion of the lexA protein, the repressor of the SOS regulon, thus inducing the SOS regulon, including the DNA repair response (81, 90, 113, 145, 151).

The recA protein is often used by investigators as the prototype for identifying the gene(s) in other organisms that are involved in the promotion of homologous pairing and strand exchange, the principal molecular events of general recombination (25, 110, 111, 126). The recombinational activity of the recA gene can conveniently be divided into three separate functions: synapsis, the pairing of homologous segments of DNA; the strand transfer or strand uptake reaction; and branch migration, the transposition of the Holliday junction along the double helix.

The synapsis reaction, like the majority of recA catalyzed reactions, requires ssDNA as a cofactor (123, 124, 150). The recA protein forms a complex with ssDNA quite readily. This complex is a much tighter one than that formed with duplex DNA (89). The binding of recA to ssDNA occurs in a cooperative manner. The optimal stoichiometry of recA to ssDNA in-vitro is 1 molecule of recA protein to 3-6 nucleotides in the presence of hydrolyzable ATP

(26, 89, 150). The result is a filamentous structure with a diameter of approximately 12nm. This structure is most likely the active species in the RecA catalyzed recombination events (eg. synapsis, strand exchange, and branch migration). That is, this 12nm filament is this structure that serves as the sequence dependent dsDNA binding entity (25, 111). RecA catalyzed synapsis follows classical enzyme kinetics indicating that the rate limiting step is a tertiary complex (112). This complex is most likely the recA-ssDNA nucleoprotein coupled to the duplex DNA in an unstable pre-D-loop structure (17, 121). The existence of such an intermediate has also been inferred by the finding that when synapsis is carried out in the presence of a non-hydrolyzable form of ATP, a filter binding intermediate consisting of DNA and protein is formed that can be dissociated by protein denaturing agents (88, 123). These results were interpreted by a model in which synapsis and strand insertion proceed via a paranemic joint (D-loop) intermediate where the strands were not interwoven around each other. Christiansen and Griffith (21) have reported finding such structures. Once the tertiary complex has been initiated, an unwinding of the duplex DNA generally follows, which is both ATP and ssDNA homology dependent (25). Minimum estimates of amount of the homology required is between 30 - 151 base pairs (42, 153). This led Cox and Lehman (25) to propose that the reaction pathway for synapsis proceeds first by a series of non-homologous reactions involving the recA-ssDNA nucleoprotein/dsDNA complex. During this stage the search for homology proceeds through an as yet

undetermined mechanism. After the duplex and single stranded DNA have been brought into homologous register, the paranemic joint (D loop) is rapidly formed. This is a necessary precursor to the ATP dependent formation of the interwoven plectonemic joint of the Holiday junction.

After synapsis has occurred, strand uptake proceeds in a polar fashion requiring ATP hydrolyses (24, 58). In the work reported by Kahn et al., chimeric duplex DNA, formed from phage M13 and phage G4 DNA sequences, exhibited a strong bias (greater than 40:1) favoring pairing initiating at the 3' end (with respect to the ssDNA) and the concurrent displacement of the helix occurring at the 5' end of the ssDNA. The polar, unidirectional nature of this strand uptake reaction would be expected to generate heteroduplex molecules. Such molecules have been observed, some longer than 1,000 bp (58, 149).

To achieve extended heteroduplex joint molecules it is necessary that a "growth" mechanism be present. This "growth" in heteroduplex DNA appears to be accomplished by the recA protein via branch migration at the rate of about 4 base pairs per second (24). Branch migration, as promoted by the recA protein with only its cofactors present, has been shown to occur past pyrimidine dimers (at a rate about 1/50 of normal). Branch migration has also been shown to occur through regions of up to 650 bp of nonhomology, past apurinic sites, and past regions of inserted or deleted DNA (10, 50, 82). To explain how recA can promote the movement of the Holliday junction along such diverse stretches of DNA as these,

analogies to other filamentous systems, such as actin, have been drawn (25). By utilizing these systems as prototypes, a model has been proposed for recA promoted branch migration. The recA-DNA filament assembled on one of the duplexes, rotates the other duplex around it driving branch migration in a unidirectional manner (27). As yet, it is still unclear what regulates the extent of branch migration or how and when Holliday junctions formed by the E. coli recA gene product are resolved (25, 120, 143).

The Bacteriophage T4 uvsX Gene And Its Product

Radding (110) addressed the central role of recA in E. coli recombination stating "...the remarkable economy with which (recA)...accomplishes both of the central steps of recombination...suggests a general mechanism based on a few principles--a mechanism that we can expect to find in operation in many systems of recombination." Thus far, Radding's expectation has been substantiated with respect to the ubiquity of the "recA function". Using assays developed during the characterization of the E. coli recA protein as the paradigm, "recA activity" has been demonstrated and often the gene coding for it has been isolated from across the breadth of living organisms. "RecA like" genes have been identified in bacterial species other than E. coli. These include Vibrio cholerae, Bacillus subtilis, Haemophilus influenzae, Proteus mirabilis, Shigella flexneri , Rhizobium meliloti, Anabaena variabilis, and Agrobacterium tumefaciens (9,

32, 35, 44, 60, 108, 122). RecA-like genes and/or recA-like proteins have been identified in yeast (3), bacteriophage T4 (154,156), the fungus Ustilago (64), mouse (both mitotic and meiotic tissue), hamster, lily (both mitotic and meiotic tissue), and human tissue or cell lines (16, 55, 61, 64). While each of these recA-like proteins has been shown to promote at least some of the same recombination activities as the E. coli recA protein, there seem to be enough differences, such as the lack of ATP dependence or variations in such parameters as Z-DNA binding affinity, to indicate that the further study of each of them will yield interesting discoveries. Of the "recA analogs" thus far identified, only the uvsX gene of bacteriophage T4 has been studied in the detail approaching that of the E. coli gene.

The uvsX gene of bacteriophage T4 was first described by Harm (45, 46) as a gene involved in the repair of UV damage and genetic recombination. Several investigators have been able to purify and characterize the uvsX gene product as well as clone the gene itself (39, 52, 154, 156). The uvsX gene product has been shown to be a ssDNA dependent ATPase with a 39,000 dalton molecular weight. It can catalyze the homologous pairing and D-loop formation reactions putting gpuvsX into the "recA-like" catagory of proteins with respect to recombination. However, there seems to be no "protease" activity associated with gpuvsX. Additionally, recombination activities of the uvsX gene product (gp) are stimulated by the presence of gp32. Gp32 has very similar single-strand DNA binding characteristics to that of ssb protein of E. coli, and appears to

interact with the gpuvsX *in vitro* in an analogous manner to the ssb interaction with the *E. coli recA* protein (25, 40). The nature of the strand transfer reactions catalyzed by recA and gpuvsX seem to be essentially the same. For example, both require a free 3' end and the transfer proceeds in the same direction in both cases. However, significant differences between the two recombinases were also observed. GpuvsX hydrolyzes ATP at a much greater rate than the recA protein and gpuvsX has a much weaker helicase activity than recA.

Genetic studies of DNA repair and recombination have indicated that the *uvsX* protein acts in the same pathway as the *uvsW* and *uvsY* gene products (8, 23, 45). To investigate the relationship between the *uvsX* and *uvsY* genes with respect to their roles in recombination, Minagawa *et al.* (96) performed a series of complementation experiments against *recA* mutants of *E. coli*. They reported that while the wild-type *uvsX* gene alone could complement *recA* mutants, it was much less efficient than wild type *recA*. However, when both the *uvsX* wild-type gene and the *uvsY* wild-type gene were added together, complementation of the *E. coli recA*$^-$ was more efficient even though gpuvsY had no detectable function when tested alone. These results strongly implicated gpuvsY as a "helper" to gpuvsX in promoting the synapsis and strand transfer reactions. These results are consistent with those reported by Formosa and Alberts (37) who found that gpuvsY has a specific affinity for binding with gpuvsX. Along with the dda gene product, gp32, gp42, an as yet unidentified host protein, and gpuvsX itself,

the gpuvsY was retained on a gpuvsX affinity column.

The Role Of Recombination In The Life Cycle Of Phage T4

While much is known about the genetic pathways and molecular mechanisms of homologous recombination, especially in E. coli and two of its phages, lambda and T4, the same cannot be said about how the recombination mechanism interacts with other macromolecular processes of the cell. As incomplete as the current state of knowledge is about the interaction of homologous recombination with other cellular processes, evidence of a pattern is beginning to develop. There seems to be a strong connection between recombination and DNA synthesis especially in the context of DNA repair. Studies of DNA repair pathways have added much to our current knowledge about the mechanics of recombination, as reflected in the models of recombination discussed earlier. The concept that "recombinational repair" can serve as a basis for "general" recombination, at least at the mechanistic level, has thus far been sustained (98). Many of the studies on the genes of phage T4 involved in DNA repair have also reported a role for these genes in recombination and DNA replication (8, 98, 102).

The potential of an intimate association between recombination and replication was noted several times in the early T4 literature (33, 46, 48, 71). In 1975, Broker and Doermann (15) proposed that T4 DNA replication was bimodal, the initial (or early) stage being responsible for the first 20 or so progeny genomes while the second

stage accounted for the remainder of the DNA synthesis (about 100 or so genome equivalents). The second stage was postulated to depend on recombination. Luder and Mosig (83, 98, 100, 101), built on this idea and proposed that the recombination dependence of the second mode was due to the replication forks initiating from recombination intermediates (this is now known as the secondary mode of initiation). Strong support for the central premise of recombinational origins has been provided by Formosa and Alberts (38) who were able to reconstitute a recombination dependent DNA synthesis system in-vitro using gpuvsX. Additional support also has been provided by Kruezer et al. (72) who have demonstrated recombination dependent replication using a plasmid based system. Bacteriophage T4's life cycle appears to be uniquely centered around recombination dependent DNA synthesis owing to the manner in which it regulates the transcription of its middle and late genes through modification of the host RNA polymerase. There are at least three modes of replication initiation reported for T4, primary, secondary, and tertiary. The primary mode of initiation of T4 DNA replication utilizes unmodified host RNA polymerase to make RNA primers (103). The secondary mode depends on recombination and was described above. The tertiary mode utilizyes neither host RNA polymerase nor recombination (93). As phage T4 infection proceeds, the host RNA polymerase is modified by T4 coded proteins so that it no longer recognizes "early promoters" including the "origin promoters" but begins transcription from "middle promoters". Once this shift has occurred, no new progeny

chromosomes can be initiated from "primary" origins of replication, which would limit burst size (fitness) if no other mode of initiation were available. Thus the T4 life cycle may have afforded recombination dependent DNA replication a unique evolutionary opportunity to maximize its efficiency (99). Does this mean that recombination dependent replication is an aberration of T4 and not to be considered any more than a evolutionary curiosity? The answer appears to be no.

In E. coli, a type of DNA replication that requires the recA gene and is recombination dependent has been shown to be an alternative initiation pathway for "normal" DNA synthesis. This pathway also explains "stable" DNA synthesis, that is synthesis in the absence of protein synthesis (66-69, 76). Additionally, Witkin et al. (152) have described a recA dependent "replisome reactivation" that seems to involve the reinitiation of DNA synthesis via a recombination intermediate. Recombination dependent DNA replication has also been demonstrated in human DNA repair systems and has been strongly implicated in the regulation of the yeast cell cycle (2, 59, 148). It is apparent that the intimate relationship between recombination and DNA replication is not just a passing fancy of phage T4 nor solely of DNA repair mechanisms per se, but that it is a recurring evolutionary theme.

Statement Of The Problem

The purpose of this study is to obtain further understanding of the interaction of the replication and recombination pathways in phage T4 and the evolutionary conservation of this interaction. Cloned bacterial *recA*$^+$ genes from *E. coli*, *A. caviea*, and *B. pertussis* and the cloned *uvsX*$^+$ gene from phage T4 will be used in complementation experiments involving an *E. coli recA*$^-$ mutant and phage T4 *uvsX*$^-$ and *uvsY*$^-$ mutants. The *B. pertussis recA*$^+$ gene was partially characterized as part of this study. The experiments will test the extent to which these "recA$^+$" gene products can substitute for the mutated gene product in recombination pathways of *E. coli* and phage T4. The *E. coli recA*$^-$ mutant will be used in a general recombination assay. Both phage mutants *uvsX*$^-$ and *uvsY*$^-$ will be used in recombination dependent DNA replication assays, and the *uvsX*$^-$ mutant in recombinational DNA repair and phage growth assays as well. The amount and pattern of complementation should provide information on both the role of the interaction between replication and recombination during the life cycle of the organism and the extent to which this role is evolutionarily conserved. In addition to the complementation experiments, an experiment will be performed to test whether a mutation in a gene associated with the phage replisome, gene42$^-$, has an effect on a recombinational repair pathway of the phage. The results of this experiment should further elucidate the nature of the interaction between replication and recombination in phage T4.

CHAPTER II

MATERIAL AND METHODS

The bacteria, phage, and plasmids used in this work are listed in Table 1. Phage strains and *E. coli* S/5/6 were obtained from the stock collection maintained by Dr. Harris Bernstein, University of Arizona. *E. coli* plasmids pSAK101, pDR3, precA35, and *E. coli* strains JC14604, HB101, and DH5 were kindly provided by Dr. Stephen A. Kuhl, University of Arizona. *E. coli* plasmid pBSK101 was obtained from Dr. Teiichi Minagawa, Kyoto University.

Media

Hershey Broth was used as described by Steinberg and Edgar (135). Bacto Nutrient Broth (Difco) was prepared using 8 g per 1000 ml and supplemented with Bacto Peptone (Difco; 5 g/L), NaCl (5 g/L), and glucose (1 g/L). The pH was adjusted to 7.2 to 7.4 with 5 N NaOH, and the medium was then dispensed and autoclaved.

M9 complete medium was prepared in accordance with Current Protocols in Molecular Biology (77). A 5x stock of M9 salts solution was prepared using Na_2HPO_4 (30.0 g/L), KH_2PO_4 (15.0 g/L), NH_4Cl (5.0 g/L), and NaCl (2.5 g/L). The pH was adjusted to 6.8 with HCl and the solution autoclaved. The M9 complete medium was made by diluting the 5x M9 stock salt solution to 1X with sterile water. Then the M9 salts solution was supplemented with the

Table 1. Bacteria, Phage and Plasmids

STRAIN	RELEVENT GENOTYPE	REFERENCE
E. coli		
S/6/5	Wild Type	
JC14604	lac⁻ MS 286081 lacBK del(srl-recA)	Clark & Marquiles (1965)
DH5	recA⁻	BRL
HB101	recA⁻	BRL
Phage		
T4D	Wild Type	
pX	uvsX⁻	Harm (1964)
y_m	uvsY⁻	Harm (1964)
Plasmids		
pBSK101	uvsX⁺ gene from phage T4	Yonesaki & Minagawa (1985)
pSAK101	recA⁺ gene from B. pertussis	Kuhl, et al. (1990)
pDR3	recA⁺ gene from A. caviea	Resnick & Nelson (1988)
precA35	recA⁺ gene from E. coli	Resnick & Nelson (1988)
pBlueScript	vector	Stratagene
pUC18	vector	BRL

following sterile solutions: 1M $MgSO_4$ (1 ml/L), 20% glucose (10 ml/L), 20% Casamino Acids (5 ml/L), and $FeCl_3$ (0.2 7mg/ml stock; 10 ml stock/L). The pH was adjusted to 7.2 to 7.4 with 5 N NaOH and the medium was then dispensed, and autoclaved.

Agar plates and soft agar (top agar) were prepared as described by Adams (135). Normal agar included Bacto Agar (Difco) at 10.0 g/L supplemented with Bacto Tryptone (Difco, 13.0 g/L), NaCl (8.0 g/L), sodium citrate (2.0 g/L), and glucose (3.0 g/L). Soft agar was made with the same ingredients as normal agar except the Bacto Agar was used at a concentration of 6.5 g/L and the glucose was reduced to 1.3 g/L.

MacConkey agar (Difco) was prepared in accordance with the directions provided by the supplier.

Phage Stock Preparation

High titer phage stocks were prepared using E. coli S/6/5 as the host. The bacteria were grown to about 5 x 10^7 cells/ml in 200 ml of Hershey broth at 37°C with aeration. Approximately 10^6 phage were then added and allowed to grow until lysis of the cell suspension was visible (5-6 hours) or overnight. A few drops of $CHCl_3$ were added to lyse any remaining cells and the cellular debris removed by centrifugation in a Sorvall GSA rotor (3,000 rpm for 15-20 minutes). The supernatant was collected and the phage pelleted in a Sorvall SS34 rotor (16,000 rpm for 2 hours). The supernatant was discarded and the pellets gently resuspended in

1-2 ml of M9 salt solution (1x). The portions were combined and the phage concentration assayed.

All phage and bacterial assays, except the in vivo recombination assay, were performed using the soft agar overlay technique (1). A dilution of bacteria or phage plus three to five drops of concentrated indicator bacteria was added to 3 ml of melted top agar. The mixture was mixed and poured onto an agar plate in an even layer. Indicator bacteria were prepared by growing 200 ml of the appropriate strain to 3-4 x 10^8 cells/ml in Hershey broth (supplemented as required). The cells were pelleted (3,000 rpm for 10-15 minutes in a GSA rotor) and resuspended in 15-20 ml of fresh media. Plates were incubated at the appropriate temperature, usually 37°C. Plaques, or bacterial colonies were counted using a New Brunswick Scientific counter. Phage stocks were stored at 4°C with 2-3 drops of $CHCl_3$. Fresh stocks were prepared when the titer dropped below 2 x 10^{10} phage/ml.

UV Sensitivity Assay

In order to determine the sensitivity of bacterial cells to ultraviolet radiation, an aliquot from a fresh overnight culture was diluted 1:100 into Hersheys Broth and the cells grown to a concentration of 1-2 x 10^8 cells/ml. A five ml aliquot of these cells was pelleted (SS34, 4000g for 5 min.), resuspended in an equal volume of M9 salts solution (1x), and stored on ice until irradiated. Irradiation was performed by exposing 1.8ml of the

washed cells in a 10 x 35 mm petri dish to the emissions of a GE Germicidal Lamp (General Electric Co., Syracuse, NY) calibrated to deliver 0.8 J/M^2. During irradiation, the cells were gently swirled and photoreactivation was prevented both during irradiation and subsequent incubation. At various intervals samples were removed and plated on Hershey agar plates using the soft agar overlay technique previously described. The plates were incubated at 37° for 14-18 hours. The colonies were counted using a New Brunswick Scientific counter. The surviving fraction was determined by dividing the bacterial titer obtained after a given dose of UV by the titer obtained at the zero dose.

Phage sensitivity to UV irradiation was measured in an analogous manner with the following differences. Phage irradiation was performed using 5.0 ml of the phage suspension at a concentration of 1-2 x 10^{10} in a 9 cm petri dish with gentle swirling. At various time intervals, a 0.5 ml aliquot was removed and diluted 10 fold into M9 salt solution (1x). These samples were stored at 4° until used. For each experiment, a fresh plating culture was prepared and the phage concentration adjusted to yield an expected multiplicity of infection (MOI) of 1 x 10^{-3}. The MOI is the ratio of phage to bacteria at the start of an experiment. Plating and incubation was done under conditions that prevented photoreactivation. Plates were incubated at the desired temperature for 14-18 hours, plaques counted using the New Brunswick counter, and surviving fraction determined.

Multiplicity Reactivation

Multiplicity reactivation experiments were performed using phage irradiated and stored as described above. Cells to be used were grown to a titer of approximately 2 - 2.5 x 10^8 cells/ml, as measured in a Petroff-Houser counting chamber. Growth was at 37°C with aeration in Hershey broth and with appropriate antibiotic selection. Cells were then washed, resuspended in an equal volume of M9 salt solution (1x), and stored on ice until used. Phage titers were adjusted to yield an estimated MOI of 1 x 10^{-3} for the monocomplex experiment and 5.0 for the multicomplex experiments.

For each experiment, an aliquot of the washed cells was taken and viable colony forming units titered by plating, using the soft agar overlay technique described above. This titer was used to calculate the actual MOI in each experiment. Phage were added at the appropriate concentration and allowed to adsorb to the cells for 10 minutes at 37°. After the adsorption period, both free phage and total infective centers were titered. Free phage were assayed by diluting first into Hershey Broth containing a few drops of $CHCl_3$, mixing vigorously to ensure killing any infected cells, and then continuing the dilution in M9 salt solution (1x). Total phage added at the start of the experiment were titered simply by diluting an aliquot in M9 salt solution (1x) and plating on Hershey plates. All plating of phage was done using the soft agar overlay technique with appropriate bacterial indicator culture. Plates were incubated at indicated temperatures for 14-18 hours and

plaques counted as described. Surviving fractions were calculated by dividing the phage titer obtained after UV irradiation, by the phage titer obtained at the zero dose of UV.

Plasmid Isolation

Plasmids were isolated from various E. coli strains using the GeneClean™ Kit (Bio101 Inc., La Jolla, CA) essentially as recommended by the supplier. Minor modifications to the boiling method were used (4). For most plasmid isolations, 1.5ml of unamplified transformed cells containing the plasmid were placed into an Eppendorf tube and the cells pelleted (30 sec. in a microcentifuge at full rpm). The supernatant was discarded and the cells resuspended in 100 ul of STET buffer (8% Sucrose, 50 mM Tris [pH 8.0], 50 mM EDTA, 5% Triton X100) containing 10 mg/ml lysozyme. The suspension was mixed and incubated on ice for at least 10 minutes. The tubes were then placed in a boiling water bath for 2 minutes followed by centrifugation in a microcentrifuge (maximum rpm) for 15 minutes at 4°. The supernatant (50-60 ul) was carefully transferred to another Eppendorf tube, without disturbing the chromosomal DNA layer, and the pellet and chromosomal DNA discarded.

The plasmid was extracted from the supernatant using Glasmilk™ as described by the supplier of the GeneClean™ Kit. To the supernatant, 2 - 3 volumes (150 - 210 ul) of NaI stock solution and 5 ul of Glassmilk™ (supplied with the GeneClean™ Kit) were

added to each tube and incubated at room temperature for 5 minutes. The Glassmilk™, now with the plasmid DNA bound to it, was pelleted in a microfuge (5 seconds at maximum rpm) and the supernatant discarded. The pellets were washed twice with 200 ul ice cold NEW solution (supplied with the GeneClean™ Kit). After the second NEW wash, the pellet was resuspended in 5 - 8 ul of deionized water or TE buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA), and heated to 50° for 3 minutes to elute off the plasmid DNA. The Glassmilk™ was pelleted in a microfuge (5 sec.) and the supernatant transferred to a fresh Eppendorf tube. A second elution was routinely performed and the supernatants combined. Plasmid DNA isolated in this manner was used directly for cloning procedures or transformation without further purification.

Preparation Of Competent Cells And Transformation

The protocol used to make E. coli cells competent was a version of the basic $CaCl_2$ protocol (4) modified by inputs from several sources including experience and "lab lore". From an overnight culture a fresh culture was started by diluting at least 10 fold into Hershey Broth, with antibiotic selection if appropriate (routinely 20 ml of cells were used per transformation). This culture was allowed to grow to approximately 5-8 x 10^8 cells/ml at which time the cells were chilled on ice for at least 10 minutes. The chilled cells were pelleted (SS34, 5,000G, 5 min.) and the supernatant discarded. The pellet was

resuspended in either 3.8 ml ice cold 0.1M $CaCl_2$ for same day use, or one tenth of the starting volume of ice cold 0.1M $CaCl_2$ for preparation of frozen competent cells. The cells were incubated on ice for at least 20 minutes after which they were competent.

The frozen competent cells were pelleted and resuspended in one-one hundredth of the starting volume of ice cold 0.1 M $CaCl_2$/glycerol (85:15). These cells were distributed in 50 ul aliquots into Eppendorf tubes and frozen as quickly as possible. The frozen cells were stored at -20° or -70° until used.

When transformations were preformed using freshly prepared cells, the cells were pipeted into four Eppendorf tubes (0.95 ml each) and the appropriate DNA added. When using frozen competent cells, the cells were first thawed on ice for 3-5 min. Then 0.9 ml of ice cold 0.1M $CaCl_2$ was added and finally the DNA was mixed in. The DNA/cell mixture prepared by either method was allowed to incubate on ice for at least 30 min. and then heat shocked at 42° - 43° for 90 seconds. The cells were then placed back on ice for an additional 2 minutes before they were pelleted (1 min. in a microfuge at maximum rpm). The transformed cells were resuspended in 1.2 -1.5 ml of Hersheys Broth without selection and allowed to incubate for 1 hour at 37° with either gentle shaking or frequent rotation. The transformed cells were then plated on to Hershey plates, with appropriate selection, by soft agar overlay or by a direct spreading technique. The plates were generally incubated at 37° for 24 - 36 hours before individual colonies were picked for analyses.

Restrictions, Ligations, Agarose Gel Electrophoresis

Restriction endonuclease digestions and ligation reactions were all performed using buffers recommended and provided by the supplier (BRL, Gathersberg, MD; IBI, New Haven, CT; Promega, Madison, WI). A typical restriction digest was performed in a 10 or 20 ul final volume containing 1-5 units of enzyme (as defined by the supplier) in a 1 ul volume. Digests were incubated at 37° for 30 minutes unless otherwise recommended by the supplier.

Ligation reactions were typically performed in a 10 ul final volume with 1-2 units of enzyme (as defined by the supplier) in a 1 ul volume. DNA and vector concentrations were optimized according to the recommendation of Legerski and Robberson (78) and the incubation carried out at 14° for 12 to 16 hours.

Agarose gel electrophoresis was performed using an agarose concentration of 0.7 - 1.0% in TBE buffer (10X stock 108 g/L Tris base, 55 g/L boric acid, 40 ml/L 0.5 M EDTA [pH 8.0] (4). Gels were typically run in a minigel apparatus (Bio-Rad, Richmond, CA) at 100v.

In Vivo Recombination Assay

In vivo recombination assays were performed essentially as described by Resnick and Nelson using the indicator E. coli JC14604 (22, 114). This strain of is useful for complementation studies of potential "recA" genes with respect to their ability to complement

a recA mutant in a homologous recombination assay. The strain is recA$^-$ with a duplication of the lac operon but with each operon having different partial deletions, one 3' and the other 5', to yield a lac$^-$ phenotype. In the presence of a "recA" function, a recombination event between the two partially deleted operons can yield a lac$^+$ papilla (detectable on MacConkey agar as a red papilla) on the colony. These papillae are counted and their frequency calculated to yield a direct measure of homologous recombination.

E. coli JC14604 was transformed with the appropriate plasmid as described above. A single colony was picked and an overnight culture grown in Hershey Broth with appropriate selection. The plasmid was reisolated from an aliquot of the transformed JC14604 as described above and its identity confirmed by restriction analyses. Once the identity of the strain was verified, a fresh culture was grown from the overnight by diluting 1:20 into Hershey broth. Appropriate selection was carried out and the cells were grown to about 3-5 x 10^8 cells/ml. Aliquots of this culture were plated onto MacConkey agar plates (Difco) so that each plate had 150 - 300 colonies on it. The plates were incubated at 37° for 48 - 50 hours at which time both the total colonies and lac$^+$ (red) papillae were determined.

DNA Synthesis Assays

Bacteriophage T4 DNA synthesis assays were performed essentially as described by Luder and Mosig (83). A fresh culture was obtained by diluting an overnight culture (grown in M9 complete medium with appropriate antibiotic selection) 10-fold into fresh M9 complete media (with antibiotic) and allowing growth to occur until a concentration of 5 x 10^8 cells/ml was obtained. At t = 0, the cultures were infected with the appropriate phage at an MOI of 6-8 and adsorption was allowed to occur for 2 minutes at 37° with constant shaking. After two minutes, t = 2, the infective centers were diluted with an equal volume of M9 complete supplemented with 20 ug/ml thymidine, 400 ug/ml of deoxyadenosine (both from Sigma), and 20 uCi of ^{3}H-thymidine (ICN, Costa Mesa, CA; specific activities of either 48.5, 58, or 64.5 Ci/mM were used). At various times after infection, 50 ul samples were taken, acid precipitated in ice cold acid precipitation solution (1 M HCl plus 0.1 M sodium pyrophosphate (4)), and stored on ice until counting.

Samples were processed by first spotting the entire sample onto a Whatman/GFA glassfiber filter that had been prewashed with ice cold ethanol and ice cold precipitation solution. After the sample was added the filter was washed three times with 3 ml of ice cold precipitation solution followed by three washings with 3 ml of ice cold ethanol. The filters were placed into a scintillation vial containing 5 ml of scintillation cocktail (Betaphlor; ICN, Costa Mesa, CA) and counted on a Beckman LS250 scintillation

counter (Beckman, Fullerton, CA).

One Step Growth Curves

The protocol used for determining one step growth curves of bacteriophage T4 was modified from that described in Adams (1). Cells to be used in the experiment were grown from fresh overnight cultures to approximately 1 x 10^8 cells/ml. One ml of cells was transferred to a 1.5 ml Eppendorf tube and the cells pelleted in a microcentrifuge (1 min. at maximum rpm). The supernatant was discarded and the cells resuspended in 1ml of M9 salt solution (1x). KCN was added to a final concentration of 0.01M and the cells incubated at 37° for 3-5 minutes. Phage were then added to an MOI of 1 x 10^{-2} and the incubation at 37° continued for 10 minutes to allow for adsorbtion. After the adsorption period, free phage were assayed as described above. At t = 0, the infective centers were serially diluted into Hershey Broth and incubated at 37°C. Total phage were assayed at various times as previously described.

Chapter III

RESULTS

Complementation Of A E. coli recA⁻ Mutant By The Cloned B. pertussis recA⁺ Gene

As part of the characterization of the newly identified recA⁺ gene of B. pertussis (74), an experiment was carried out to test whether the cloned recA⁺ gene of B. pertussis complements an E. coli recA⁻ mutant as measured by resistance to UV radiation. The results depicted in Figure 4 represent the survival, upon UV irradiation, of E. coli RR1 (recA⁺) transformed with the vector pUC18 without insert as a wild type control, HB101 (recA13) transformed with pUC18 without insert as a negative control, and HB101 transformed with pBSK101 carrying the cloned recA gene from Bordetella pertussis. The cells were irradiated as described using a UV dose of 0.8J/M² for the times indicated. The data indicate that the B. pertussis recA⁺ gene is able to complement the recA13 allele with respect to repair of UV induced DNA damages. The B. pertussis gene decreased the rate of UV inactivation by 5-fold.

Complementation Of An E. coli recA⁻ Mutant By Cloned "recA⁺" Genes In A General Recombination Assay

To investigate the evolutionary conservation of the "recA" function within general recombination, three "recA" genes were examined for their ability to complement an E. coli recA⁻ mutant in a general recombination assay. Each of the cloned "recA" genes was independently transformed into E. coli JC14604 as described in Materials and Methods. Table 2 presents the results of these experiments comparing the recombination proficiency between E. coli JC14604 transformed with the vector pBlueScript (pBS) without insert as the negative control, pRecA35 carrying the cloned E. coli recA⁺ gene, pDR3 carrying the cloned Aeromonas caviae recA⁺ gene, pSAK101 carrying the B. pertussis recA+ gene, and with pBSK101 carrying the uvsX⁺ gene of phage T4. In these experiments, the A. caviae recA⁺ gene complemented the E. coli recA⁻ mutant at a level equal to 58% of the cloned wild type E. coli recA⁺ gene, a value similar to that reported by Resnick and Nelson (114), while both the B. pertussis recA⁺ gene, and the phage T4 uvsX⁺ gene complemented at the 2% level. The results in Table 2 imply that the B. pertussis recA protein and the phage T4 uvsX protein catalyze the same central reactions of homologous recombination as the E. coli recA protein but at a much lower efficiency.

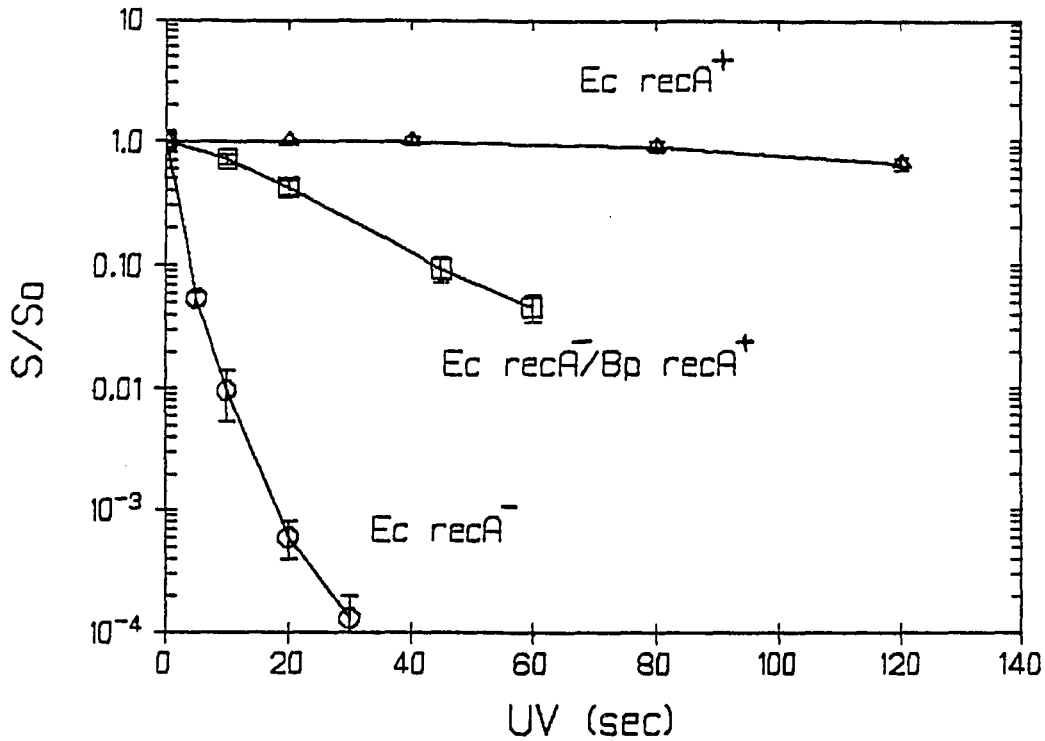

Figure 4. Survival of E. coli Strains After UV Irradiation. The abbreviations used are: Ec recA$^+$ = E. coli RR1 (recA$^+$) transformed with pUC18 without insert; Ec recA$^+$/Bp recA$^+$ = E. coli DH5 (recA$^-$) transformed with pSAK101 (B. pertussis recA$^+$); Ec recA$^-$ = E. coli DH5 (recA$^-$) transformed with pUC18 without insert.

Table 2. In vivo Recombination Frequencies Between Genetic Markers in an E. coli recA⁻ Mutant *

Plasmid	recA Gene	Total Colonies	Recombinants	%Recomb.*
pBS	None	2810	0	0
pRecA35	E. coli recA⁺	1221	949 (.777)	100
pDR3	A. caviae recA+	1333	603 (.452)	58
pSAK101	B. pertussis recA⁺	4005	58 (.014)	2
pBSK101	phage T4 uvsX⁺	1438	21 (.015)	2

* E. coli JC14604 (recA⁻) was the recipient in all plasmid transformations.

* The % recomb. is the percent recombination expressed relative to the frequency of recombination in E. coli JC14604 (recA⁻)/precA35 (recA⁺).

Complementation Of A Bacteriophage T4 uvsX⁻ Mutant By Various Bacterial recA⁺ Genes With Respect To Mode 2 DNA Replication

To begin studying the question of whether or not the "recA" function can be complemented in recombination dependent DNA synthesis by other "recA" gene products, I performed the following series of DNA synthesis experiments. The cloned recA⁺ genes from either E. coli, A. caviae, or B. pertussis were inserted into E. coli DH5 by transformation, as described in Materials and Methods, and each was tested to determine if it could complement a phage T4 uvsX⁻ mutation in recombination dependent DNA replication. The results of these experiments are given in Figures 5-9. Figure 5 depicts phage DNA synthesis measured as described in Materials and Methods. Phage T4D (wt) and phage px (uvsX⁻) were the infecting phage strains and E. coli DH5 (recA⁻) transformed with pBS without insert was the host. DNA synthesis was approximately the same in both wild type and mutant for the first 15 minutes. However, while wild type shifted phage DNA synthesis to a rapid linear mode, phage px (uvsX⁻) displayed the DNA synthesis arrest phenotype previously described (30, 91). The curves of DNA synthesis shown in Figure 5 were used as a basis of comparison to the results of the various complementation experiments to be described. Figure 6 shows that the phage px mutant (uvsX⁻) is complemented by the wild type phage T4 uvsX⁺ gene carried on pBSK101 in the host E. coli DH5 (recA⁻). The uvsX⁺ gene on a plasmid causes a shift to a linear mode of DNA synthesis that has an essentially identical slope as the wild type.

Thus, the DNA arrest phenotype is complemented by the cloned uvsX$^+$ gene. However, the initial phase of DNA synthesis in the presence of the cloned uvsX$^+$ gene is significantly slower than in either the wild type infection or the infection by the mutant alone.

Figures 7 through 9, respectively, provide the same comparison as in Figure 6 for complementation experiments between the phage uvsX$^-$ mutant and each of the three cloned bacterial recA$^+$ genes. The data for the complementation of the phage uvsX$^-$ mutant by the E. coli recA$^+$ gene, carried on pRecA35 and transformed into DH5, is given in Figure 7. The initial phase of phage DNA synthesis of the phage px mutant grown in DH5 (pRecA35) is essentially identical with the wild type. However there is also a clear complementation of the uvsX$^-$ mutation by the E. coli recA$^+$ gene during the later stage of DNA synthesis. The data for the complementation of the phage uvsX mutant by the recA$^+$ gene of A. caviae, carried on pDR3 in E. coli DH5 is shown in Figure 8. This bacterial recA gene complements the DNA arrest phenotype of the phage mutation to essentially wild type levels. It is worth noting that DNA synthesis by the phage px grown in DH5 (pDR3) shows only a very brief delay compared to the wild type phage. Figure 9 shows the results of testing the B. pertussis recA$^+$ gene for its ability to complement the uvsX$^-$ mutant in the phage DNA synthesis assay. The B. pertussis recA$^+$ gene was cloned in pBSK101 using E. coli DH5 (recA$^-$) as host. The B. pertussis recA$^+$ gene strongly complemented the DNA arrest phenotype of the phage uvsX$^-$. The resulting rate

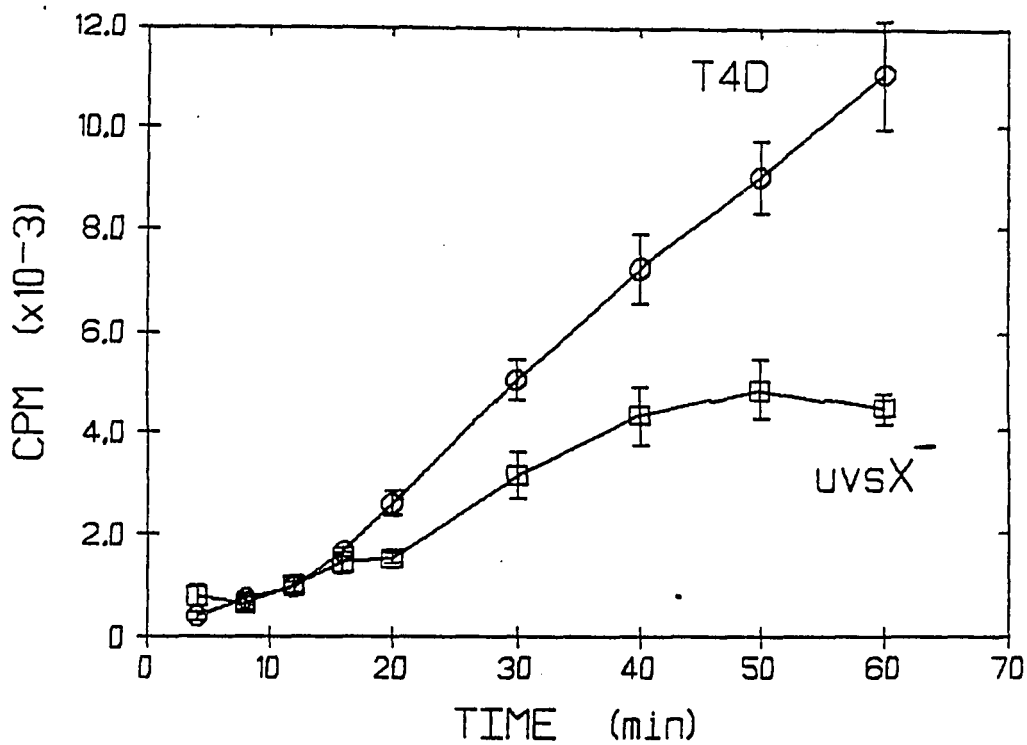

Figure 5. DNA Synthesis of Phage T4. Abbreviations used are: T4D = T4D (wt phage T4) DNA synthesis in E. coli DH5 (recA⁻) transformed with pBS (no insert); uvsX = phage px (uvsX⁻ phage T4) grown in E. coli DH5 (recA⁻) transformed with pBS (no insert). Error bars indicate the standard error in four experiments.

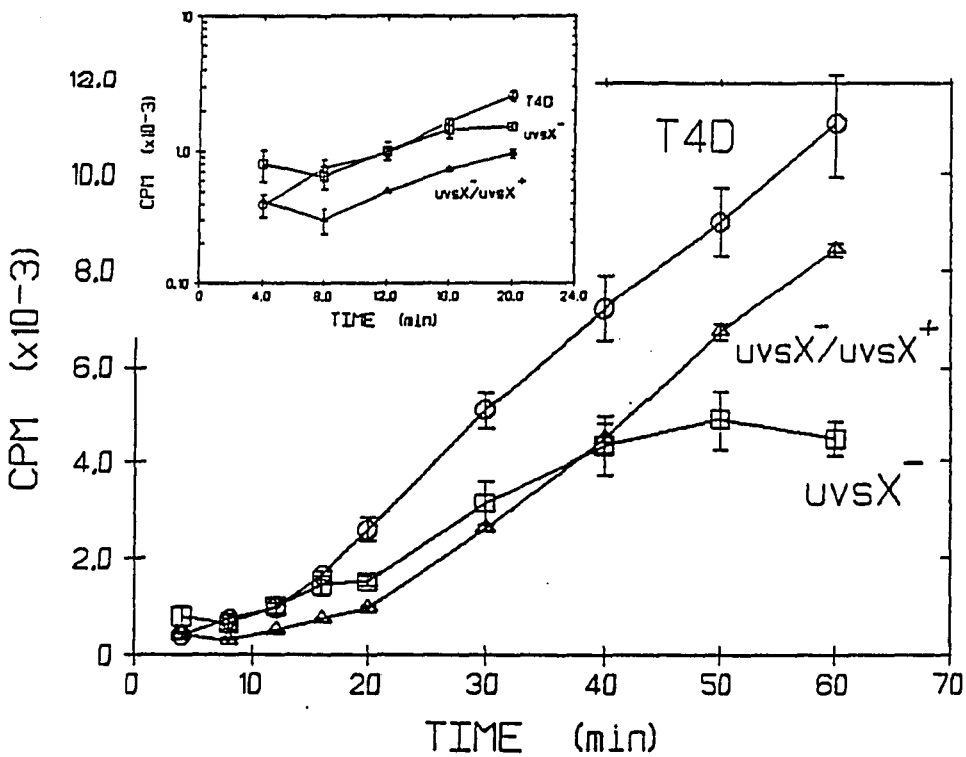

Figure 6. DNA Synthesis of Phage T4. Curves labeled T4D and uvsX are taken from Figure 5. uvsX⁻/uvsX⁺ = phage px (uvsX⁻) grown on the host E. coli DH5 (recA⁻) transformed with pBSK101 which contains the phage uvsX⁺ gene. Each point on the uvsX⁻/uvsX⁺ curve represents the mean of three experiments.

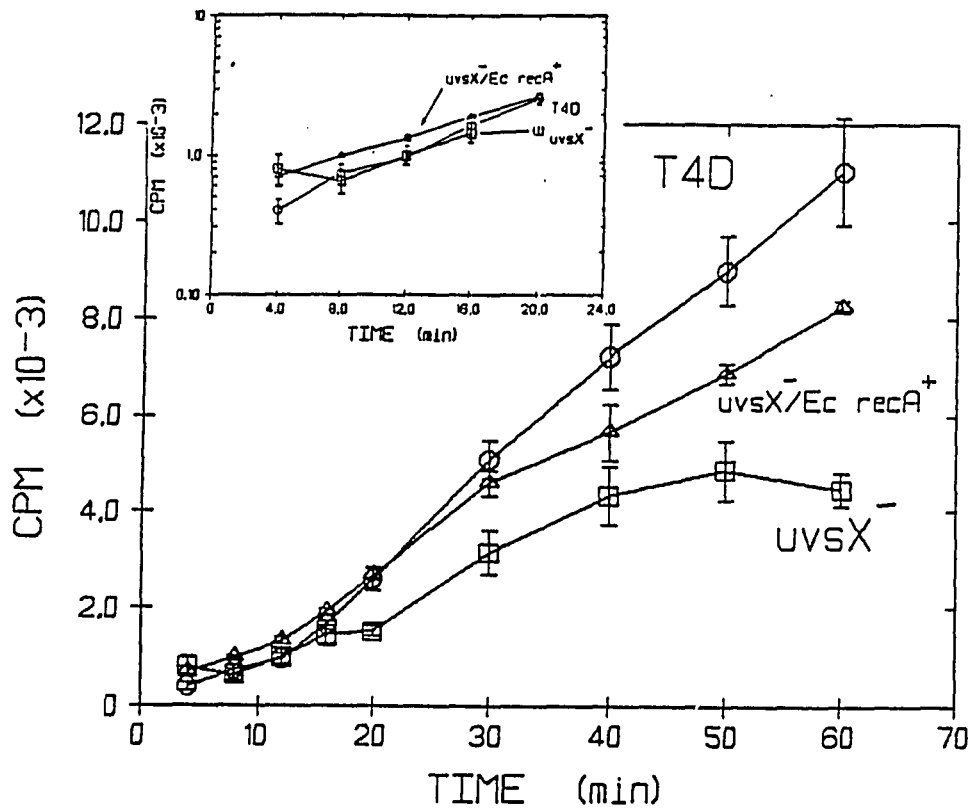

Figure 7. DNA Synthesis of Phage T4. Curves labeled T4D and uvsX are taken from Figure 5. uvsX⁻/Ec rēcA⁺ = phage px (uvsX⁻) grown on the host E. coli DH5 (recA⁻) transformed with pRecA35 which contains the E. coli recA⁺ gene. Each point on the uvsX⁻/Ec recA⁺ curve represents the mean of three experiments.

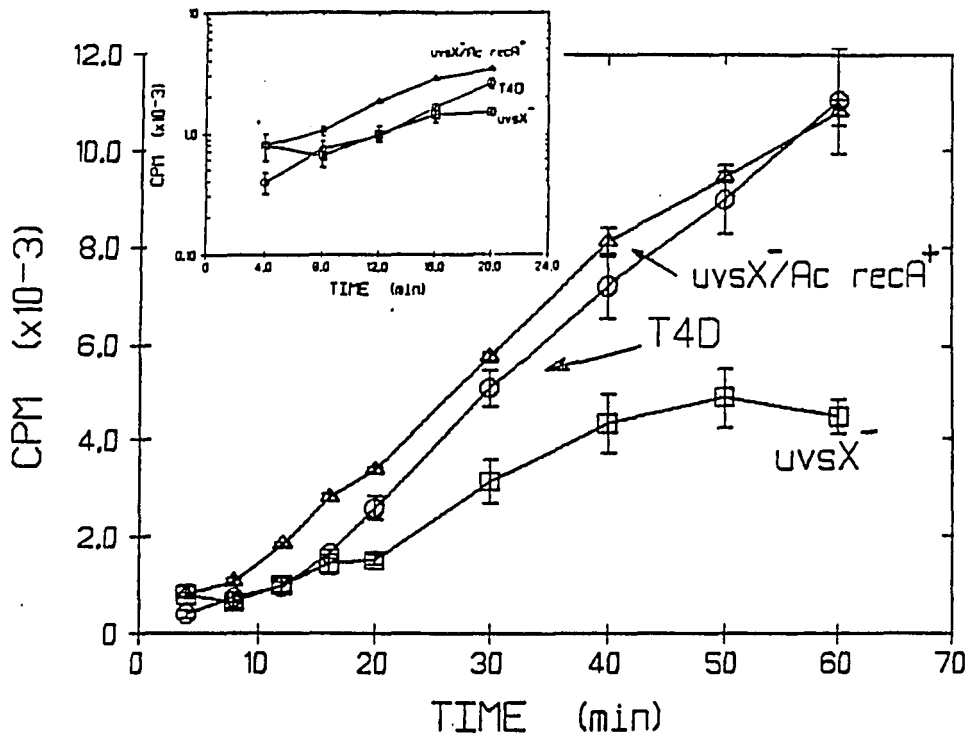

Figure 8. DNA Synthesis of Phage T4. Curves labeled T4D and uvsX are taken from Figure 5. uvsX⁻/Ac recA⁺ = phage px (uvsX⁻) grown on the host E. coli DH5 (recA⁻) transformed with pDR3 which contains the A. caviae recA⁺ gene. Each point on the uvsX/Ac recA⁺ curve represents the mean of three experiments.

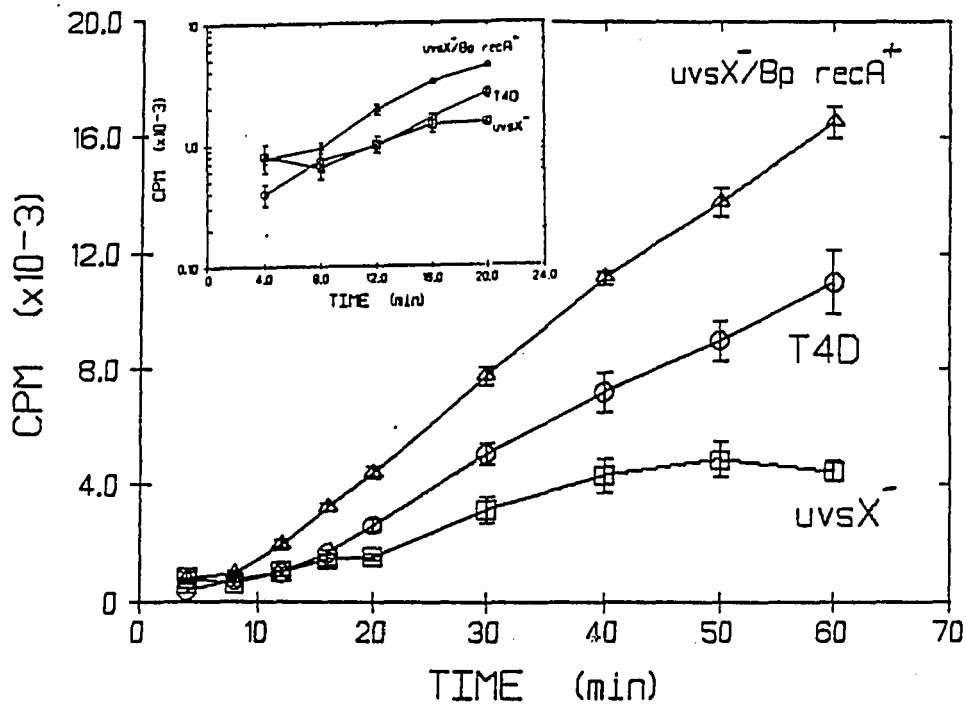

Figure 9. DNA Synthesis of Phage T4. Curves labeled T4D and uvsX are taken form Figure 5. uvsX⁻/Bp recA⁺ = phage px (uvsX⁻) grown on the host E. coli DH5 (recA⁻) transformed with pSAK101 which contains the B. pertussis recA⁺ gene. Each point on the uvsX⁻/Bp recA⁺ curve represents the mean of three experiments.

of DNA synthesis substantially greater than that of the wild type phage. As was observed in the A. caviae experiments (Figure 8), there is only a very brief delay prior to the phage entering the linear phase of DNA synthesis when the uvsX$^-$ mutant is complemented by the B. pertussis recA$^+$ gene.

Complementation Of A Phage T4 uvsY$^-$ Mutant By Various Bacterial recA Genes With Respect To Mode 2 DNA Replication

To further address the question on the nature of the interaction between replication and recombination in phage T4, the question was asked, "Would the cloned bacterial recA$^+$ genes also complement a uvsY$^-$ mutant phage in Mode 2 DNA replication?" This experiment had the added aspect of observing the effect of having both the E. coli recA$^+$ gene and the phage T4 uvsX$^+$ gene present together. The DNA synthesis assay, previously described, was used to examine the ability of the E. coli, A. caviae, and B. pertussis recA$^+$ genes to complement the phage T4 uvsY$^-$ mutant y_m. Figure 10 presents the results of the experiments for phages T4D (wt) and y_m (uvsY$^-$) with E. coli DH5 (recA$^-$) transformed with pBlueScript (without insert) used as the host. In these experiments the uvsY$^-$ mutant displays a DNA arrest phenotype (30, 91). In Figures 11 through 13 the results of the complementation experiments are given. In each series of experiments, the phage was grown using E. coli DH5 (recA$^-$)

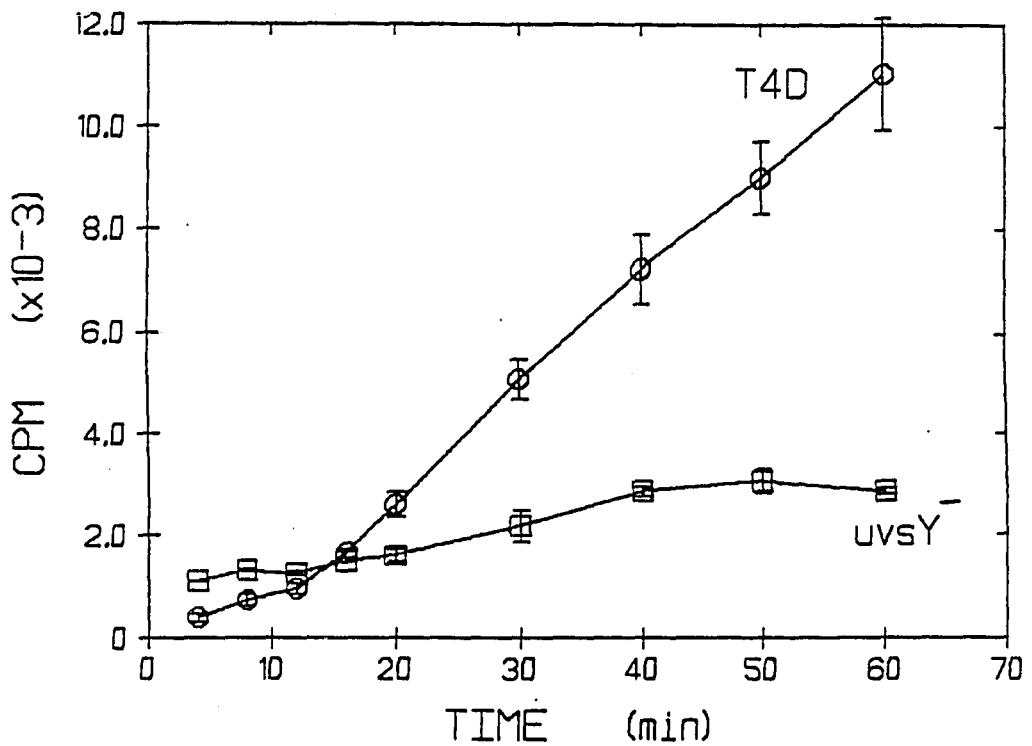

Figure 10. DNA synthesis of Phage T4. Abbreviations used are: T4D = phage T4D (wt) grown in E. coli DH5 (recA$^-$) transformed with pBS (no insert); uvsY = phage y_m (uvsY$^-$) grown in E. coli DH5 (recA$^-$) transformed with pBS (no insert). Each of the points in the curves represent the mean of three experiments.

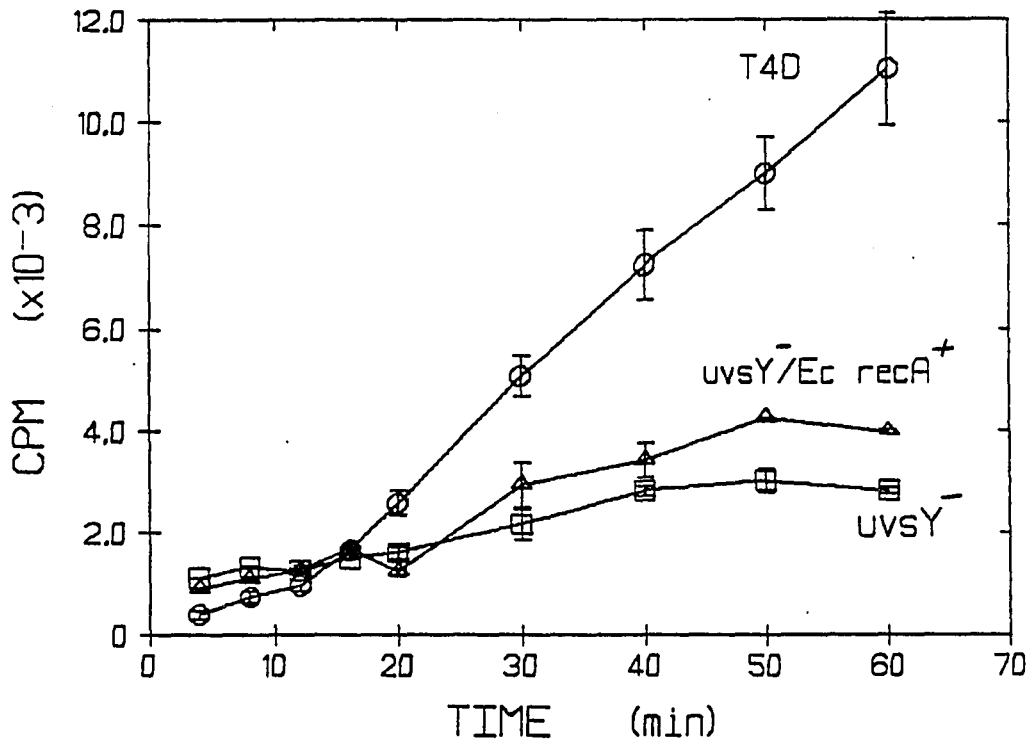

Figure 11. DNA Synthesis of Phage T4. Curves labeled T4D and uvsY are taken from Figure 10. uvsY⁻/Ec recA⁺ = phage y_m (uvsY⁻) grown in E. coli DH5 (recA⁻) transformed with pRecA35 which contains the E. coli recA⁺ gene. Each of the points in the curve uvsY⁻/Ec recA⁺ represents the mean of three experiments.

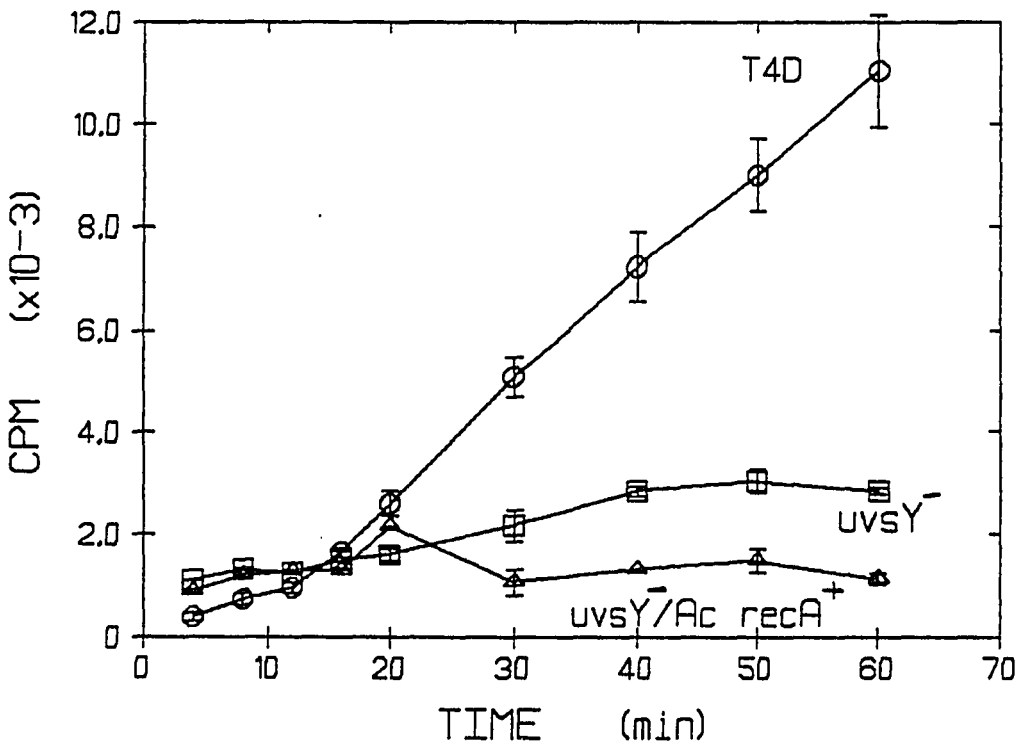

Figure 12. DNA Synthesis of Phage T4. Curves labeled T4D and uvsY are taken from Figure 10. uvsY⁻/Ac recA⁺ = phage y_m (uvsY⁻) grown in E. coli DH5 (recA⁻) transformed with pDR3 which contains the A. caviae recA⁺ gene. Each of the points in the the uvsY⁻/Ac recA⁺ curve represents the mean of three experiments.

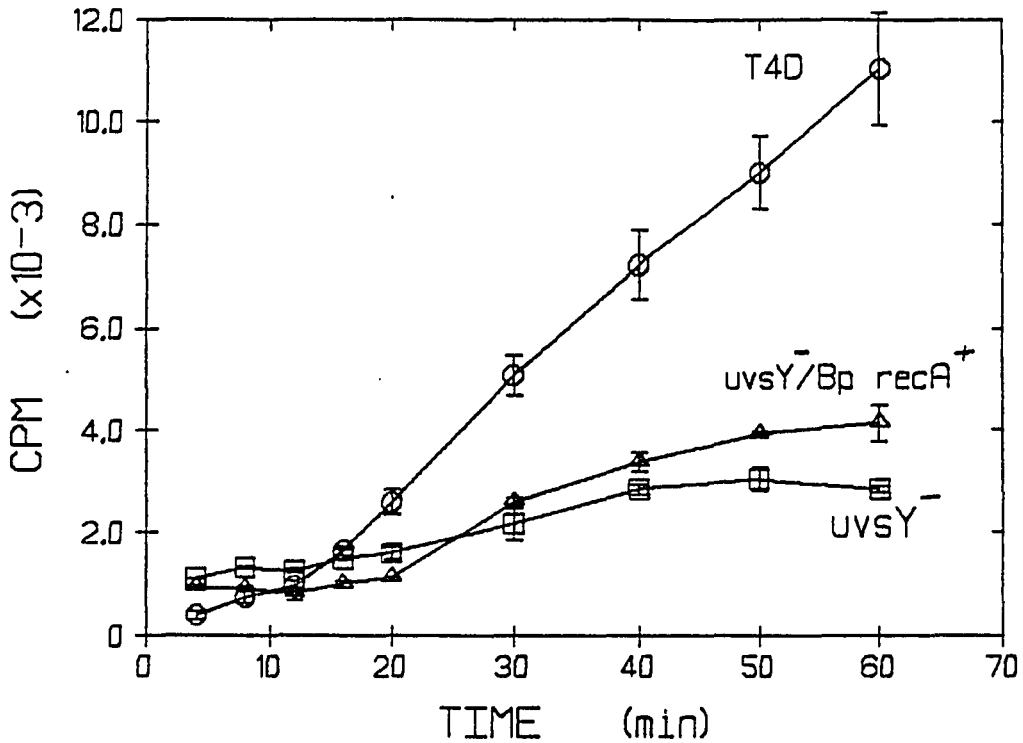

Figure 13. DNA Synthesis of Phage T4. Curves labeled T4D and uvsY are taken from Figure 10. uvsY⁻/Bp recA⁺ = phage y_m (uvsY⁻) grown in E. coli DH5 (recA⁻) transformed with pSAK101 containing the B. pertussis recA⁺ gene. Each of the points in the curve labeled uvsY⁻/Bp recA⁺ represents the mean of three experiments.

transformed with the plasmid carrying the indicated recA$^+$ gene. As can be seen, none of the bacterial recA$^+$ genes substantially complemented the DNA arrest phenotype of phage uvsY$^-$ mutation. In fact, while the course of DNA synthesis in the presence of the E. coli and B. pertussis recA$^+$ genes were slightly greater than that of the uvsY$^-$ mutant alone, the A. caviae recA$^+$ gene appeared to inhibit DNA synthesis of the uvsY$^-$ mutant phage.

Complementation Of The Phage uvsX$^-$ Mutant By Various Bacterial recA$^+$ Genes In A UV Survival Assay

To examine the effect of the cloned bacterial recA$^+$ genes on the DNA repair capabilities of a uvsX$^-$ phage, UV survival experiments were performed using the px (uvsX$^-$) phage. The data presented in Figures 14 through 18 were obtained using the UV sensitivity assay described in Materials and Methods utilizing E. coli DH5 (recA$^-$) transformed with plasmids containing the different bacterial recA$^+$ genes (see Table 1). In Figure 14, the data for phage T4D (wt) and phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pBlueScript without insert is presented. The survival curve of phage px displays the previously reported UV sensitive phenotype characteristic of uvsX$^-$ mutations (45). The effect of complementation of the cloned uvsX$^+$ gene (carried on pBSK101) is presented in Figure 15 along with the data from Figure 14 for comparison. As can be seen, the cloned uvsX$^+$ gene complements the px phage and confers a near wild type resistance to

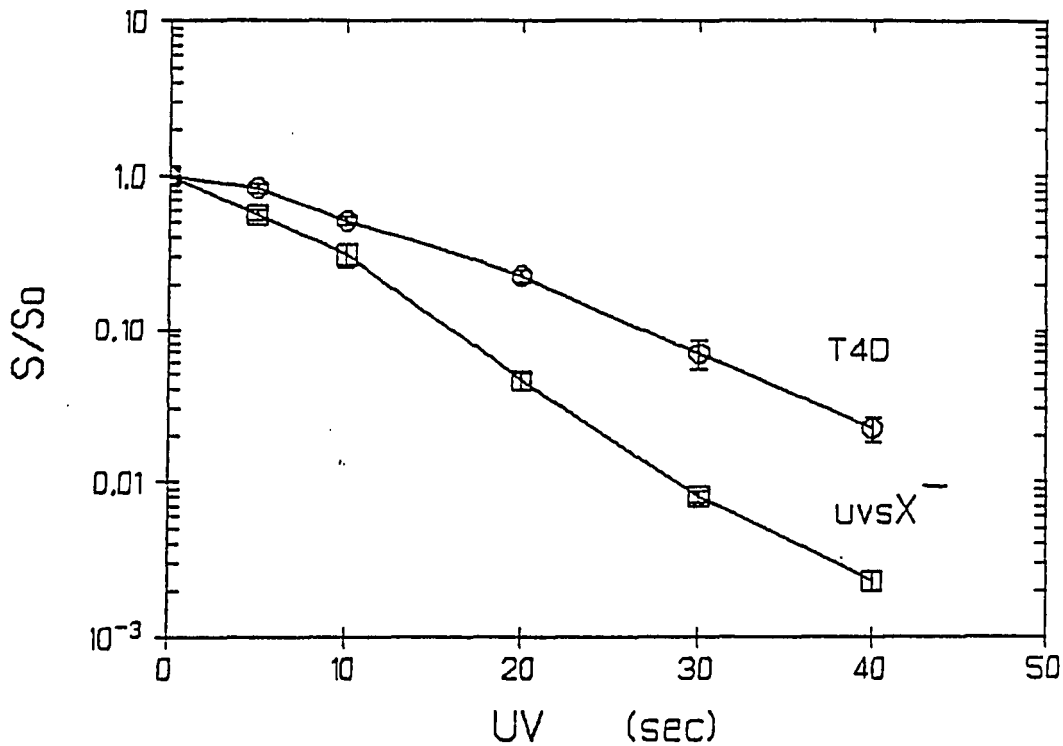

Figure 14. Survival of Phage T4 Strains After UV Irradiation. Abbreviations used are: T4D = phage T4D (wt) grown in E. coli DH5 (recA⁻) transformed with pBS (no insert); uvsX⁻ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pBS (no insert). Each point in each curve represent the mean of three experiments.

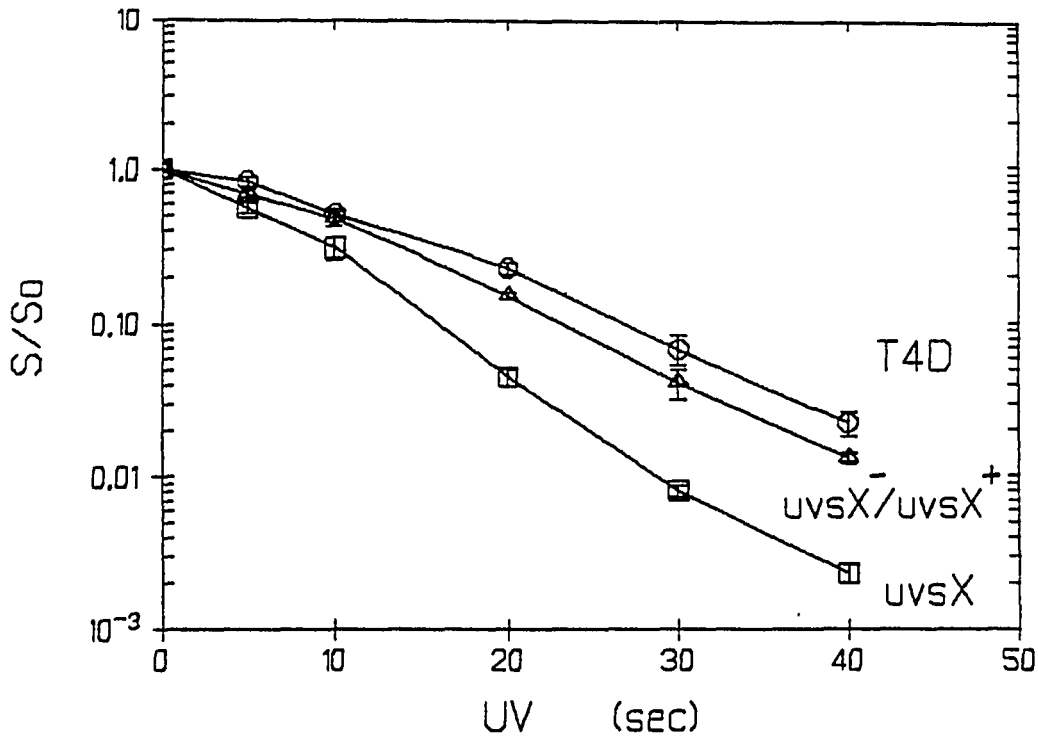

Figure 15. Survival of phage T4 Strains After UV Irradiation. Curves labeled uvsX⁻ and T4D are taken from Figure 14. uvsX⁻/uvsX⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pBSK101 containing the uvsX⁺ gene. Each point in the uvsX⁻/uvsX⁺ curve represents the mean of three experiments.

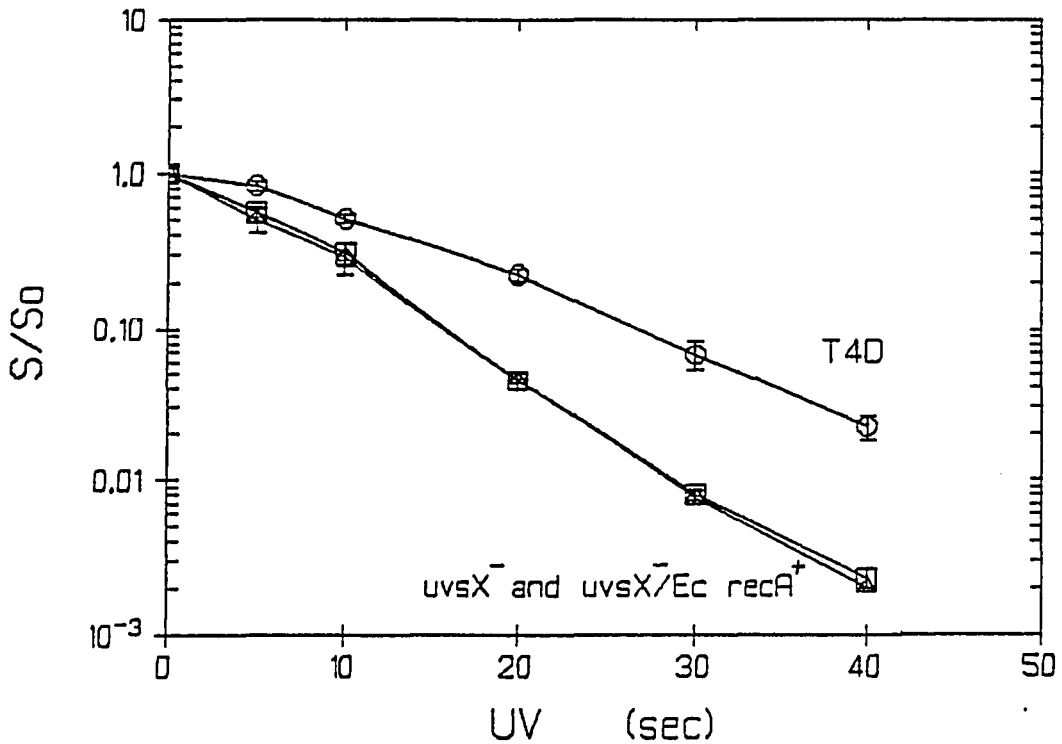

Figure 16. Survival of Phage T4 Strains After UV Irradiation. Curves labeled uvsX⁻ and T4D are taken from Figure 14. uvsX⁻/Ec recA⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pRecA35 which contains the E. coli recA⁺ gene. Each point in the uvsX⁻/Ec recA⁺ curve represents the mean of three experiments.

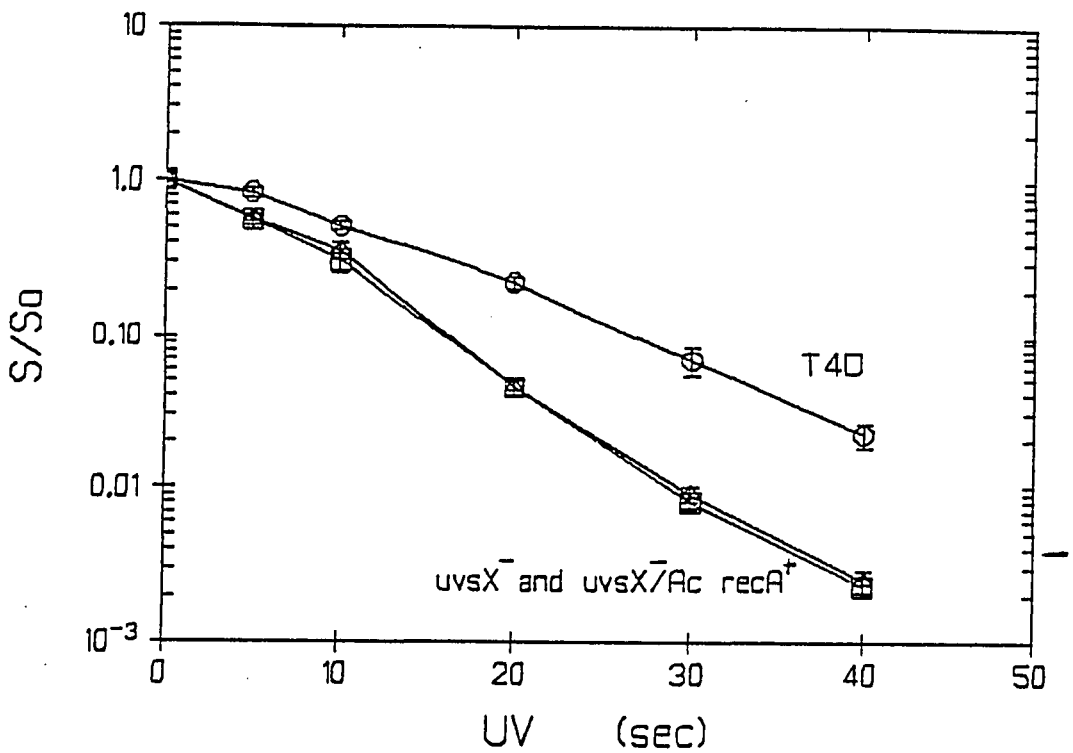

Figure 17. Survival of Phage T4 Strains After UV Irradiation. Curves labeled uvsX⁻ and T4D are taken from Figure 14. uvsX⁻/Ac recA⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pDR3 which contains the A. caviae recA⁺ gene. Each point in the uvsX⁻/Ac recA⁺ curve represents the mean of three experiments.

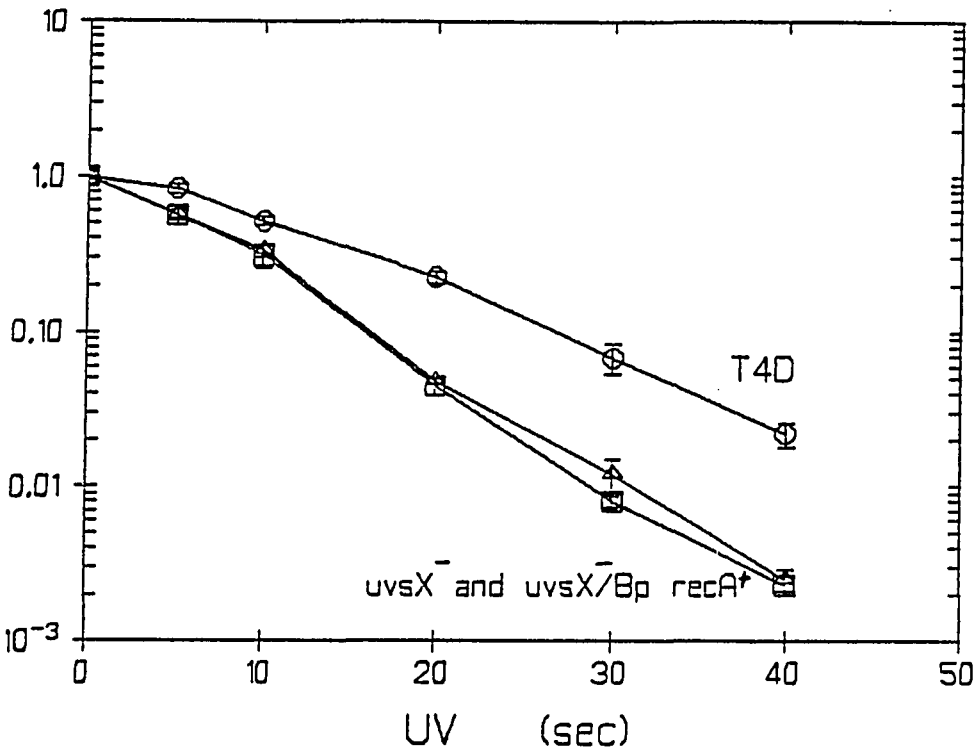

Figure 18. Survival of Phage T4 Strains After UV Irradiation. Curves labeled uvsX⁻ and T4D are taken from Figure 14. uvsX⁻/Bp recA⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pBSK101 which contains the B. pertussis recA⁺ gene. Each point in the uvsX⁻/Bp recA⁺ curve represents the mean of three experiments.

UV irradiation. The results of the complementation experiments using the cloned bacterial recA$^+$ genes are given in Figures 16 through 18 along with the curves from Figure 14 for comparison. None of these recA$^+$ genes were able to complement the phage px (uvsX$^-$) mutant with respect to resistance to UV irradiation.

One Step Growth Curves

In order to determine the effect of the various cloned bacterial recA genes on the growth of a uvsX$^-$ mutant, a series of one step growth curve experiments were performed as described in Materials and Methods. The results from a representative series of experiments are shown in Figures 19 through 23. Table 3 represents the burst size calculated from each of the growth curves depicted in Figures 19 - 23. Figure 19 shows the growth curves of phages T4D (wt) and px (uvsX$^-$) when both were grown in E. coli DH5 (recA$^-$) transformed with pBlueScript without any insert. The growth curve of the mutant displays a longer latent period and a smaller burst size than that of the wild type. The latent period is the period between the initial infection and the start of the rise period in the one step growth curve. Figure 20 depicts the growth curve of the uvsX$^-$ phage grown in E. coli DH5 (recA$^-$) transformed with pBSK101 (uvsX$^+$) compared to the curves shown in Figure 19. The complementation of the uvsX$^-$ mutation by the cloned wild type uvsX gene results in the restoration of an approximately normal latent period and, as can be seen in Table 3, in a slightly

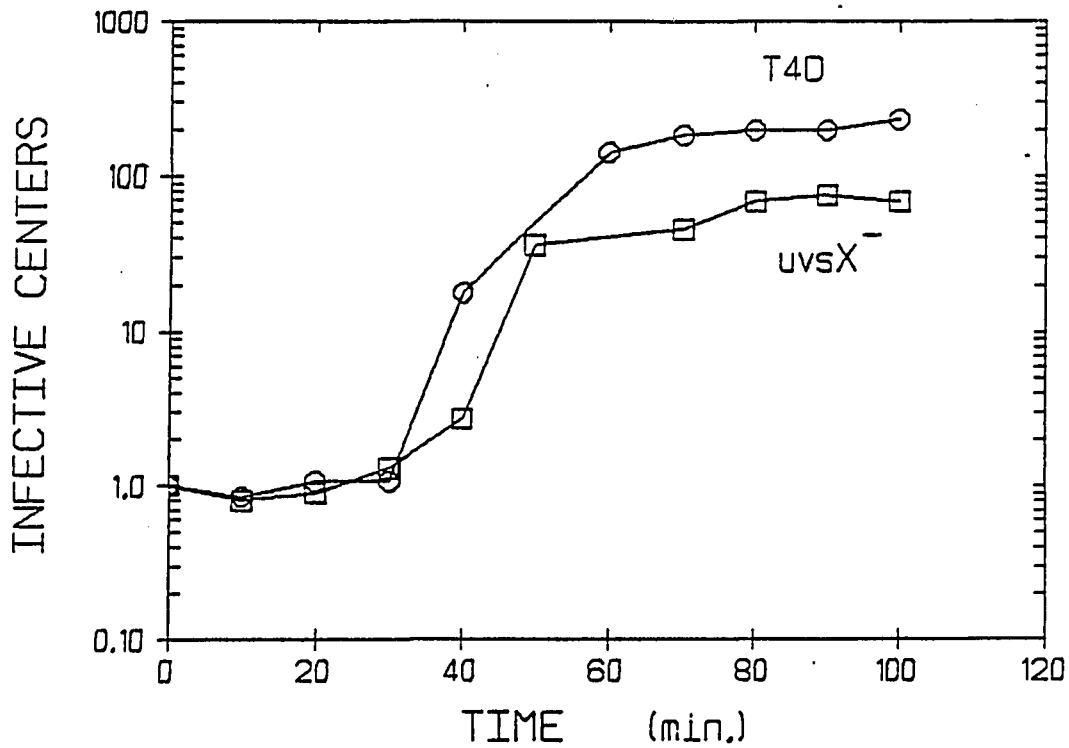

Figure 19. One Step Growth Curves. Abbreviations used are: T4D = phage T4D (wt) grown in E. coli DH5 (recA-) transformed with pBS (no insert); uvsX- = phage px (uvsX-) grown in E. coli DH5 (recA-) transformed with pBS (no insert).

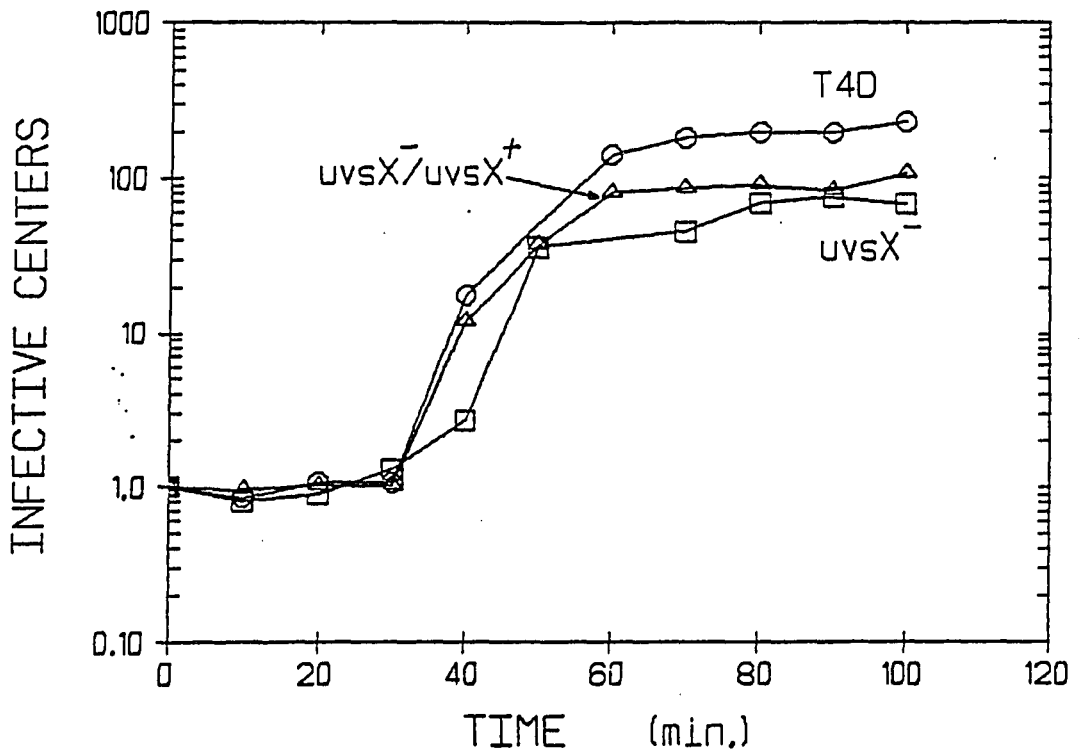

Figure 20. One Step Growth Curves. Curves labeled T4D and uvsX- are taken from Figure 19. uvsX⁻/uvsX⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pBSK101 containing the phage uvsX⁺ gene.

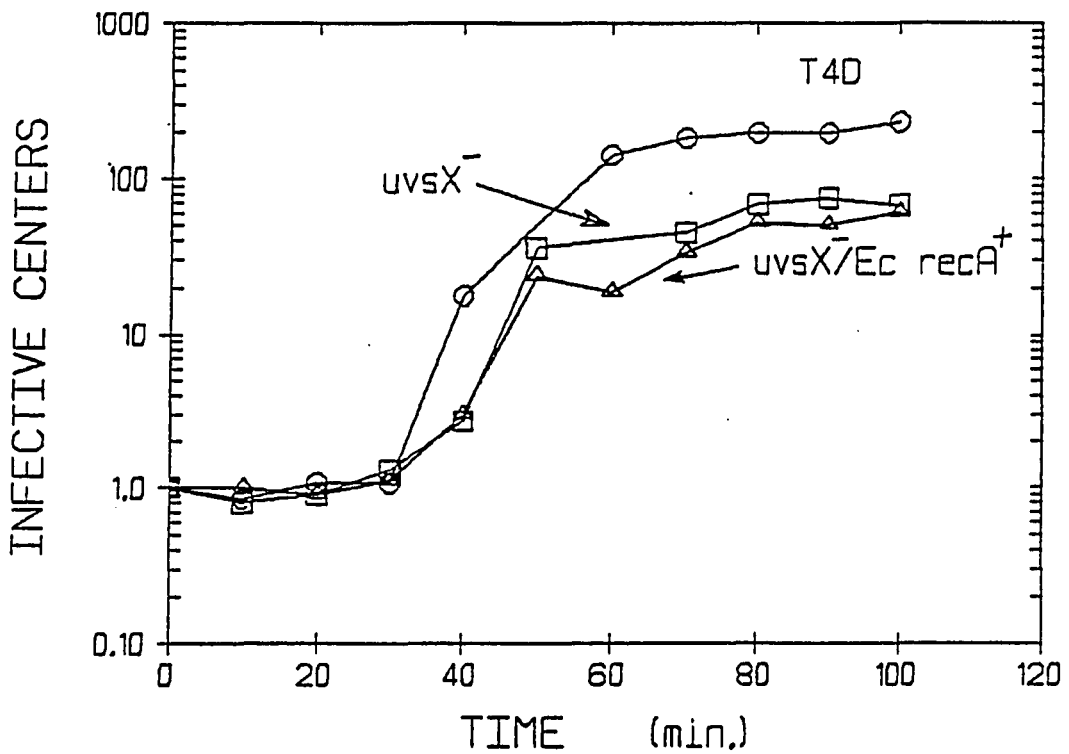

Figure 21. One Step Growth Curves. Curves labeled T4D and uvsX⁻ are taken from Figure 19. uvsX⁻/Ec recA⁺ = phage px (uvsX⁻) grown in E. coli DH5 (recA⁻) transformed with pRecA35 containing the E. coli recA⁺ gene.

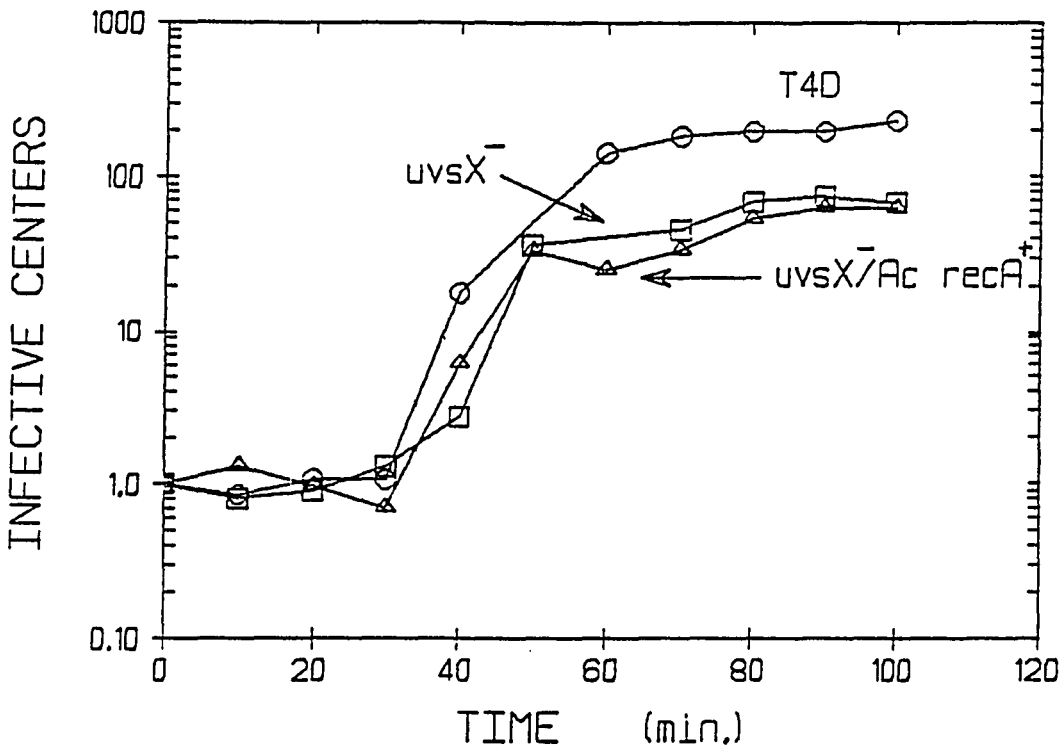

Figure 22. One Step Growth Curves. Curves labeled T4D and uvsX- are taken from Figure 19. uvsX-/Ac recA+ = phage px (uvsX-) grown in E. coli DH5 (recA-) transformed with pDR3 containing the A. caviae recA+ gene.

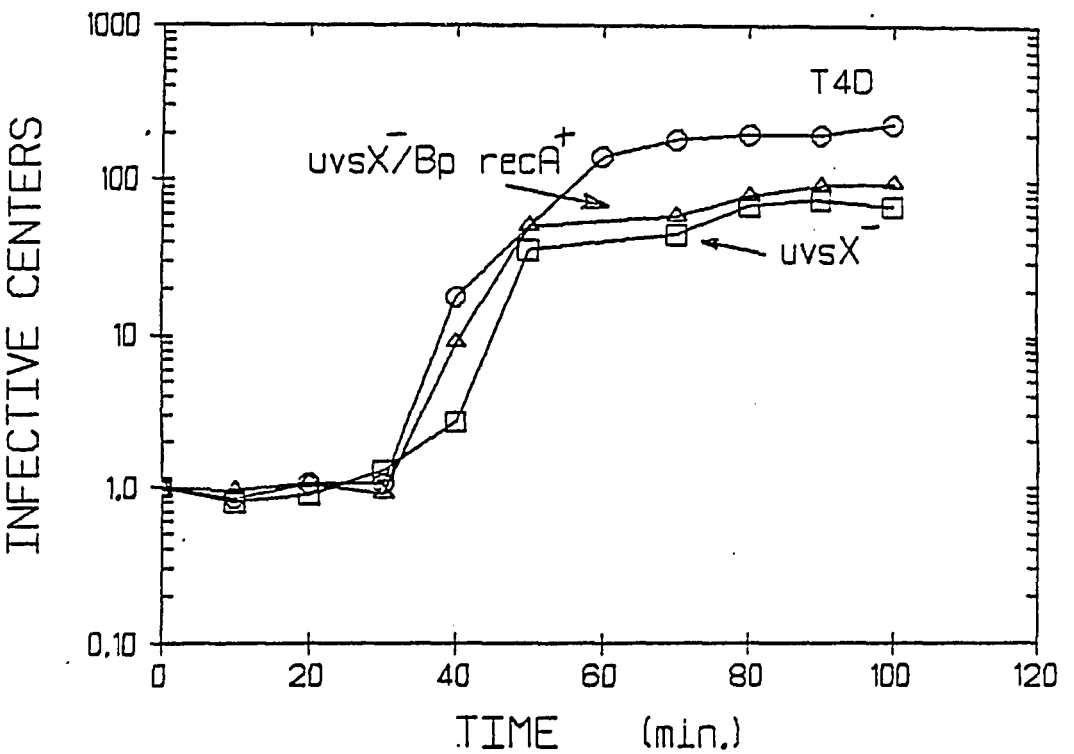

Figure 23. One Step Growth Curves. Curves labeled T4D and uvsX- are taken from Figure 19. uvsX-/Bp recA+ = phage px (uvX-) grown in DH5 (recA-) transformed with pSAK101 which contains the B. pertussis recA+ gene.

Table 3. Burst Size

Phage	Burst Size*	Fraction of WT Burst Size
T4D	190	1.00
uvsX$^-$	70	0.37
uvsX$^-$/uvsX$^+$	93	0.49
uvsX$^-$/Ec recA$^+$	54	0.28
uvsX$^-$/Ac recA$^+$	60	0.32
uvsX$^-$/Bp recA$^+$	87	0.46

Abbreviations used are: T4D = phage T4D (wt) grown in E. coli DH5 (recA$^-$) transformed with pBS (no insert); uvsX$^-$ = phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pBS (no insert); uvsX$^-$/uvsX$^+$ = phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pBSK101 which contains the phage uvsX$^+$ gene; uvsX$^-$/Ec recA$^+$ = phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pRecA35 which contains the E. coli recA$^+$ gene; uvsX$^-$/Ac recA$^+$ = phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pDR3 which contains the A. caviae recA$^+$ gene; uvsX$^-$/Bp recA$^+$ = phage px (uvsX$^-$) grown in E. coli DH5 (recA$^-$) transformed with pSAK101 which contains the B. pertussis recA$^+$ gene.

* Burst size was calculated from the data presented in Figures 19 - 23 an d represents the number of infective centers after 70 minutes of infection divided by the number at t = 0.

increased burst size (from 70 to 93 phage/cell). The effect of the cloned recA$^+$ genes from E. coli and A. caviae on the growth of phage px in E. coli DH5 are shown in Figures 21 and 22 and Table 3. Neither of these bacterial recA$^+$ genes complemented the uvsX mutation phage px with respect to the latent period and both slightly depressed the burst size compared to the infection by the uvsX$^-$ mutant alone. The effect of the cloned B. pertussis recA$^+$ gene can be seen in Figure 23 and Table 3. When the uvsX$^-$ phage was grown in E. coli DH5(pBSK101), the B. pertussis recA$^+$ gene restored the latent period to near that of wild type and elevated the burst size as well (from 70 to 87 phage/cell). In each of the three series of experiments performed, the relative effect on the growth of the mutant px phage was exactly as described above, though there was a large variation in the absolute numbers between each series.

Multiplicity Reactivation With A Gene 42 Mutant

The gene 42 product is dCMP-hydroxymethylase which is known to be associated with the replicative enzyme complex (85). I tested whether the enzyme is necessary for recombinational repair in order to determine whether there is a coupling between the replication and recombinational repair complexes. Multiplicity reactivation experiments were conducted as described in the Materials and Methods section using UV irradiated phage tsL66 (42$^-$). The results of these experiments are shown in Figure 24.

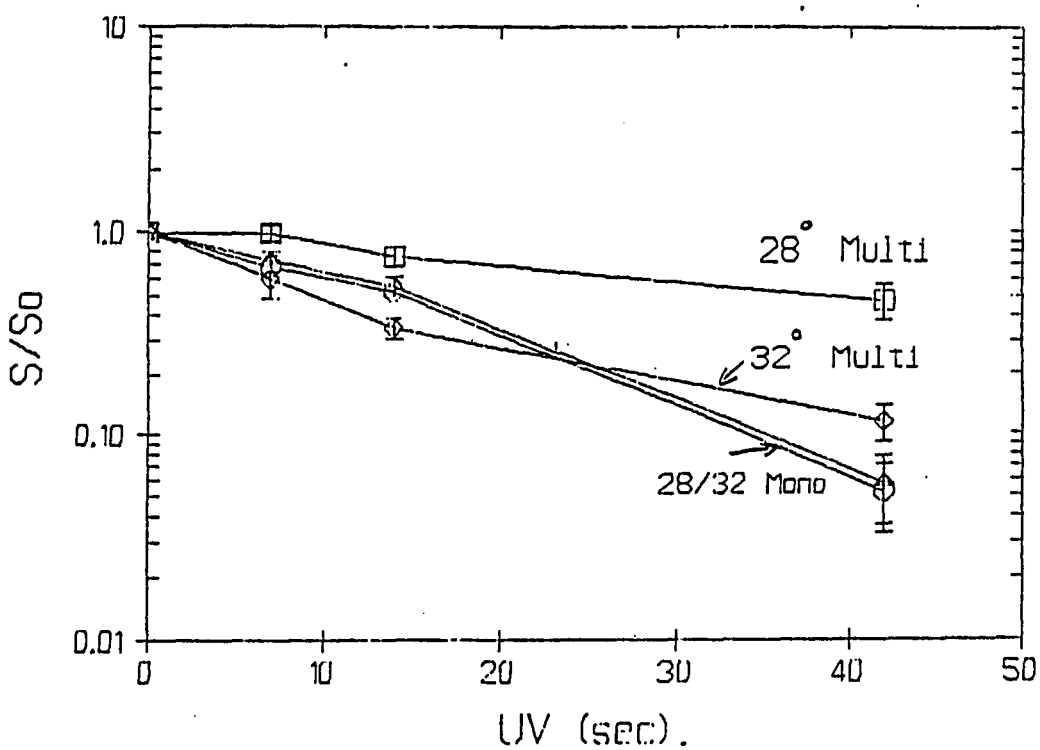

Figure 24. MR by a Gene 42ts Mutant. Abbreviations used are: 28° Multi = phage tsL66 (42-) grown at 28°C with an MOI of 5; 32° Multi = phage tsL66 (42-) grown at 32°C with an MOI of 5; 28° Mono = phage tsL66 (42-) grown at 28°C with an MOI of 10^{-3}; 32° Mono = phage tsL66 (42-) grown at 32°C with an MOI of 10^{-3}. Each curve represent the mean of three experiments.

When the experiments were conducted at 28°C, suppressing the temperature sensitive mutant phenotype, the effect of recombinational repair could be seen as the difference in survival between the multicomplexes and the monocomplexes. When the experiments were conducted at 32°C, allowing for partial expression of the mutant phenotype, there was no difference in the survival of the monocomplexes compared to the experiments conducted at 28°C. Moreover, the survival of the multicomplexes was only slightly better than that of the monocomplexes implying that there was little or no recombinational repair occurring under these experimental conditions.

Chapter IV

DISCUSSION

B. pertussis recA Protein Functions

As disucssed in the Introduction, the "recA function" appears to be ubiquitous. Thus identifying the recA gene equivalent in any organism is a reasonable starting point in the investigation of the basic DNA metabolism of the organism. The present study focuses on a comparison if the "recA: function of phage T4 to the "recA" functions of several bacteria. First, a putative recA$^+$ gene from Bordetella pertussis was isolated and characterized (74). As part of that study, I performed complementation experiments to determine the extent of functional homology between the recA gene of E. coli and the putative recA gene of B. pertussis with respect to the repair of UV induced DNA damage and general recombination.

The B. pertussis recA gene was able to effectively complement an E. coli recA mutation with respect to the repair of UV induced DNA damages (Figure 4). However, it was relatively ineffective in complementing the general recombination function (Table 2). Putative recA genes from two other organisms were also tested for their ability to complement the E. coli recA$^-$ mutant. They were the A. caviae recA$^+$ gene and the phage T4 uvsX$^+$ gene. The data from the general recombination assay imply that these recA genes fall into two categories. The cloned A. caviae recA complemented the E.

coli recA mutation in JC14604 approximately 58% as well as the cloned wild type E. coli recA gene, a value slightly higher than that reported by Resnick and Nelson (114). However, both the B. pertussis recA gene and the phage T4 uvsX gene complemented at approximately the 2% level. The similar behavior of the phage T4 and B. pertussis genes in complementing the E. coli mutant with respect to homologous recombination suggests that these two genes, while catalyzing the same homologous pairing and strand insertion reactions of homologous recombination, are less closely related functionally to the E. coli recA$^+$ gene than the A. caviae recA$^+$ gene. Minagawa et al. (96) reported no increase in survival, when the cloned phage T4 uvsX$^+$ gene was introduced into an E. coli recA$^-$ mutant and then subjected to low doses of UV irradiation (less than 1 J/M^2). Only slight (less than 2 fold) complementation was obtained at higher doses. Thus the finding that phage T4 uvsX$^+$ shows little complementation of an E. coli recA$^-$ mutant with respect to recombination (Table 2) is in agreement with the results of Minagawa et al. who measured complementation in terms of DNA repair. Possible explanations for these findings will be discussed below.

uvsX Complementation By Bacterial recA Genes In Mode 2 Synthesis

As described in detail in the Introduction, phage T4 has at least three distinct modes of replication initiation. The so called secondary mode (or Mode 2) is recombination-dependent and it

is eliminated by mutations in phage genes which are essential for recombination such as gene uvsX. The goal of these experiments was to determine if the "recA function" from several bacterial species could complement the uvsX gene of phage T4 with respect to recombination-dependent DNA replication (Mode 2 DNA synthesis). Therefore to test if cloned bacterial recA$^+$ genes could complement a phage uvsX$^-$ mutant a series of complementation experiments were carried out using the phage DNA synthesis assay described by Luder and Mosig (83). The time course of DNA synthesis of phage T4D (wild type) and of phage px (uvsX$^-$) are given in Figure 5. The curves obtained from both phages are consistent with those previously reported using similar assays (30, 83, 86, 91). The T4D curve is biphasic with the initial exponential DNA synthesis being followed by a linear phase that is prolonged under the conditions of the assay. The DNA synthesis curve of the uvsX$^-$ phage shows the DNA arrest phenotype described by Cunningham and Berger (1977). The initial rate of DNA synthesis appears essentially identical with that of wild type but the px phage is not able to make the shift to the linear mode. This dependence of continued phage DNA synthesis on the uvsX$^+$ gene product was used by Mosig to describe Mode 2 or the "secondary" mode of DNA synthesis (98). Mode 2 DNA synthesis has been shown to require not only the uvsX gene product but the products of phage genes uvsY, 46, 59, 39, and 52 as well (72).

When phage px was grown in the presence of the cloned uvsX$^+$ gene, the ability of the px mutant to shift to the linear

mode of DNA synthesis was restored. This is evident in Figure 6 as the portion of the curve after approximately 20 minutes post infection that is linear and parallel to the same portion of the T4D (wt) curve (the curves presented in Figure 5 were redrawn for comparison in Figures 6 through 9). This implies that the DNA arrest phenotype of the uvsX$^-$ mutant is complemented by the cloned wild type uvsX$^+$ gene. The initial exponential rate of DNA synthesis, however, is depressed by the presence of excess gpuvsX$^+$ early on in the infection and the shift to the linear DNA synthesis mode is delayed somewhat. A possible explanation for this is that the gpuvsX$^+$ produced by the cloned gene binds to the parental phage forming DNA/protein filaments which interfere with the functioning of the primary origins of replication thus slowing the initial rate of DNA synthesis (134). Additionally it should be noted that the experiments were done under conditions of high multiplicity of infection. This condition along with the over-production of gpuvsX$^+$ should have provided ample opportunity for the immediate utilization of mode 2 DNA replication. However, the phage did not implement this mode of replication until after a significant amount of "normal" DNA synthesis had occurred. This suggests that the shift to Mode 2 DNA synthesis is a regulated event in the life cycle of the phage.

Next, the cloned recA$^+$ genes from E. coli, A. caviae, and B. pertussis were tested for their ability to complement the uvsX$^-$ mutant with respect to Mode 2 DNA synthesis. The E. coli recA$^+$ gene product promoted the same initial DNA synthesis rate as T4D

(wt) and the uvsX⁻ mutant (Figure 7). However, it partially complemented the DNA arrest phenotype of the uvsX⁻ mutant. This implies that the E. coli recA gene can partially provide the recombination intermediate utilized by the phage as a mode 2 origin of replication. However, these origins are apparently either too few in number or are not as efficiently used by the phage DNA replication complex as those formed by gpuvsX⁺. While the E. coli RecA⁺ protein forms complex DNA protein filaments as does gpuvsX⁺ (134), there was no detectable effect, either enhancement or suppression (as was noted with gpuvX⁺), of the initial phage DNA synthesis.

One possible explanation of the partial complementation by the E. coli RecA⁺ protein is that there is a reduction in the amount of this protein during the course of the infection. This reduction could be due to a change in transcription rates caused by phage modifications of the E. coli RNA polymerase, to decreased protein half-life during the infection, or some combination of these events. This possibility was addressed by Minagawa et al. who reported that the concentration of the E. coli RecA protein did not change during the course of a phage infection (96).

The A. caviae recA⁺ protein was able to fully complement the DNA arrest phenotype of the uvsX⁻ mutants allowing essentially the wild type rate of linear synthesis until approximately 60 minutes post infection (Figure 8). This suggests that the A. caviae RecA⁺ protein provides a recombination-dependent primer equivalent to that provided by gpuvsX⁺. Futhermore, A. caviae RecA⁺ protein does

this in a quantity and/or quality more consistent with that in the wild type T4D infection than the cloned E. coli RecA$^+$ protein. A stimulatory effect of the A. caviae RecA$^+$ protein on the initial rate of DNA synthesis is also evident and is consistent with the phage being able to utilize the Mode 2 origins immediately to achieve an overall enhancement of initial DNA synthesis (Figure 8). As discussed above, this was the result that was expected, but not observed, when complementation was by the wild type gpuvsX$^+$.

The results of the complementation between the B. pertussis RecA protein and the uvsX mutation clearly show greater than 100% complementation (Figure 9) . These results suggest that, as with the E. coli and A. caviae RecA$^+$ proteins, the B. pertussis RecA$^+$ protein provides the phage a recombination-dependent origin of replication equivalent to that provided by the wild type uvsX$^+$ protein. The A. caviae and the B. pertussis data are consistent with an immediate utilization of the Mode 2 origins by the phage's replication "machinery".

The extent of the complementation by B. pertussis recA$^+$ implies that the regulatory mechanism controlling the rate of phage DNA synthesis has been largely bypassed, so that the level of DNA synthesis attained may well be close to the maximum DNA replication potential of phage T4. This presumptive regulatory bypass may be explained by either of two possibilities. First, the B. pertussis RecA$^+$ protein may be present in such a relatively high concentration that it is able to provide so many Mode 2 origins that the regulatory element(s) are in effect "overwhelmed" rather

than bypassed. Alternatively, the B. pertussis RecA$^+$ protein may be fully capable of complementing the role of gpuvsX$^+$ in forming stable Mode 2 origins at essentially wild type levels but incapable of interacting directly with any of the proteins of the replisome that participate in the regulation of DNA synthesis. Thus the data may reflect a true "bypass" of the regulatory mechanism. These two alternatives will be addressed in more detail below.

That at least some bacterial "recA" genes do complement the role of the uvsX gene in Mode 2 DNA synthesis is consistent with the reports of several different phage and bacteria (such as T7, lambda, and B. subtilis) having a recombination dependent mode of DNA synthesis (69, 76, 98). It can therefore be hypothesized that the linkage between replication and recombination seen in phage T4 also occurs in E. coli, A. caviae, and B. pertussis as well. If this is the case, it is reasonable to suspect that the coupling is accomplished using the same intermediates, for example priming from a "D loop", as described for the phage system.

uvsY Complementation By Bacterial recA Genes In Mode 2 Synthesis

The uvsY gene of phage T4 belongs to the same repair and recombination pathway as the uvsX gene. Although no specific function has yet been determined for its gene product, it appears to have at least some of the same properties as the E. coli recA gene product (8, 30). Recent studies by Minagawa and his coworkers (96) indicate that gpuvsY augments the expression of all of the

"recA" functions of gpuvsX. In order to determine if either the E. coli, A. caviae, or the B. pertussis RecA proteins could complement a uvsY$^-$ mutation with respect to its DNA arrest phenotype, a series of complementation experiments were performed. The time course of DNA synthesis by the uvsY$^-$ mutant is shown in Figure 10 along with that for the wild type. The phenotype of the mutant is consistent with that reported by Cunningham and Berger (30) in that it involves a more complete arrest of DNA synthesis than that of a uvsX$^-$ mutant. As can be seen in Figures 11 through 13, none of the bacterial RecA$^+$ proteins significantly complemented the DNA arrest phenotype of the uvsY$^-$ mutant. In fact the A. caviae RecA$^+$ protein essentially shuts down DNA synthesis after the point where the phage is relying on Mode 2 initiation (Figure 12). Additionally, there was no detectable effect of the bacterial recA genes on the initial phase of DNA synthesis as was seen in the uvsX experiments.

When the results of the uvsY and uvsX experiments are taken together, a clearer picture emerges to explain the effect of the bacterial recA$^+$ genes, especially the one from B. pertussis, on phage recombination-dependent DNA replication. In the uvsX$^-$ experiments, the genotype of the phage was uvsX$^-$, uvsY$^+$ and the DNA arrest phenotype was not only complemented by the B. pertussis recA$^+$ gene (Figure 9), but the rate of DNA synthesis was substanially increased compared to the wild type phage infection. In the uvsY$^-$ experiments, the phage genotype was uvsX$^+$, uvsY$^-$ and there was neither complementation of the DNA arrest phenotype nor any increase in synthesis rate. The amount of bacterial recA gene

expression should have been essentially the same in both types of experiments since the same cell/plasmid combinations and identical growth conditions were used in all experiments. If the increase in DNA synthesis was due solely to the increased amount of recA protein available, then one would expect to see the same effect in the uvsY experiments as in the uvsX series. In as much as this is not the case, the presence or absence of the uvsX protein is likely the determining factor.

In the experiments with the y_m phage (uvsY$^-$), the wild type gpuvsX$^+$ was present, although not able to fully catalyze the reactions of homologous recombination without the uvsY protein (96). It has been reported that the wild type uvsX protein interacts with at least ssb, dsDNA, ssDNA, and itself *in vitro*. The uvsX protein also catalyzes "D-loop" formation even in the absence of the uvsY$^+$ protein (38-40, 96, 154-156). It is reasonable to suppose that this is also true *in vivo* under the conditions of these experiments. The bacterial RecA proteins, as seen in the experiments with the uvsX$^-$ mutant (uvsX$^-$, uvsY$^+$; Figures 6-9), each were capable of sustaining at least some Mode 2 DNA replication in the absence of functional gpuvsX. Additionally, the presence of gpuvsY has been shown to have no effect on the recombination functions of the *E. coli* recA protein (96, 155). Therefore, the failure of the bacterial "recA" genes to complement the y_m phage (uvsY$^-$, uvsX$^+$) with respect to Mode 2 phage DNA replication (Figures 11-13) cannot be satisfactorilly explained as an inactivation of either the gpuvsX or the bacterial recA

proteins. Since in the uvsX$^+$, uvsY$^-$ experiments the wild type bacterial "recA" genes were present and in a form capable of providing functional Mode 2 origins of replication, the failure to complement uvsX$^+$, uvsY$^-$ phage probably reflects a problem of regulation rather than one of enzymatic function. As the wild type gpuvsX was present, it should have been capable of interacting with double and single stranded DNA and other phage proteins including, presumably, those involved in phage DNA replication. Thus the replisome might preferentially interact with the recombinational origins formed by gpuvsX. This could, in effect, "blind" the phage replisome to any Mode 2 origins generated simultaneously by the bacterial RecA proteins. In this situation the defective gpuvsY made by the uvsY$^-$ mutant would block replication from Mode 2 origins unless complemented by one of the bacterial RecA proteins. However, the results obtained show that such complementation does not occur. In light of this interpretation, the excess DNA synthesis observed in the uvsX$^-$/*B. pertussis* recA$^+$ infections (Figure 9) is most simply explained by a lack of interaction between the *B. pertussis* RecA protein and the phage regulatory components which down regulate Mode 2 DNA synthesis during phage infection. This lack of inhibition would lead to "runaway replication".

There are two general catagories of data in the literature that may also be explained by the hypothesis of inefficient protein-protein interaction offered above. First, it has been shown that the *E. coli* RecA protein does not effectively substitute

for gpuvsX in such assays as *in vitro* Mode 2 DNA synthesis, DNA repair, and *in vitro* recombination (38, 96, 97, 155). Secondly, the presence of both gpuvsX and gpuvsY in a wild type *E. coli* cell interferes with the functioning of gprecA as measured by increased *E. coli* UV sensitivity (96, 155). To date no completely satisfactory explanation for these findings has been offered. The most widely held view, that the highly modified nature of the native T4 DNA (glycosylated hydroxymethyldeoxycytosine containing DNA) rendered the *E. coli* RecA protein essentially useless in phage metabolism, never addressed the second set of data and was effectively refuted by Yonesaki and Minagawa with respect to the first (155). However, their explanation for the interference between the *E. coli* recA protein and gpuvsX and gpuvsY, that the gprecA is "inactivated" during a phage infection, is not consistent with the data presented in this study.

The alternative I propose is that during a phage infection, the *E. coli* *recA* protein may well remain present and fully functional with respect to its recombination activities. However, the bacterial protein may not be able to fully interact with the phage proteins, thus prohibiting its enzymatic activity from being integrated into the phage's recombination or replication pathways. This explanation would fit all of the instances where gprecA has not been able to substitute for gpuvsX. A simular argument can be made for the interference of gprecA by gpuvsX and gpuvsY. When present in the wild type *E. coli*, the phage proteins, gpuvsX and gpuvsY, would be expected to act efficiently on the DNA to form

recombination intermediates. However, these phage-produced intermediates might not be recognized, and therefore be unusable, by the bacterial proteins in the recombination and repair pathways due to inappropriate protein-protein interaction. This would be true even though, at the DNA level, the DNA-protein intermediates formed by gpuvsX and gpuvsY would be analogous to those formed by gprecA. The existence of a significant number of "unusable" recombination intermediates in the UV damaged bacterial cell would undoubtably reduce the efficiency of the recombinational DNA repair system assayed by Yonesaki and Minagawa (155). I will further address this issue of competing protein interactions below.

uvsX Complemented By Bacterial recA Genes In A UV Survival Assay

In order to examine whether or not a uvsX$^-$ mutant px could be complemented by the recA genes from E. coli, A. caviae, or B. pertussis in a DNA repair pathway, a series of UV survival experiments were preformed. As can be seen from the data in Figures 14 and 15, the cloned uvsX$^+$ gene could complement the uvsX mutant. However, as shown in Figures 16 through 18, none of the bacterial RecA proteins were able to complement the function of gpuvsX in the repair of UV induced DNA damages. The experiments were performed using a multiplicity of infection of less than one. Thus the repair pathway being looked at was postreplication recombinational repair (PRRR) which has been shown to depend on gpuvsX (ie. recombination) (8). For recombinational repair to

occur under conditions of infection where only one phage chromosome is in each host, the phage must accomplish both DNA replication and recombination in concert for the phage to survive. This might require near maximal integration of the phage's replication and recombination pathways. The data clearly indicate that, under these experimental conditions, only the wild type gpuvsX could function appropriately.

One Step Growth Curves

To examine the effect of the three cloned bacterial recA$^+$ genes during the normal growth of a uvsX$^-$ mutant, a series of one step growth curves were performed. Representative results are shown in Figures 19 through 23 and Table 3. The uvsX$^-$ mutant exhibited the extended latent period and reduced burst size (Figure 19 and Table 3)previously reported to be characteristic of the mutant (45, 46). The cloned wild type uvsX$^+$ gene complemented the mutant phenotype (Figure 20 and Table 3) with respect to latent period and burst size. However, while the latent period was complemented to essentially that of wild type, the burst size was only increased from 37% of wild type in the case of the uvsX$^-$ infection to 49% of wild type in the uvsX$^-$/uvsX$^+$ infection. The recA gene products from both E. coli and A. caviae did not complement either component of the mutant phenotype. (Figures 21, 22 and Table 3) The B. pertussis recA gene perhaps complemented the uvsX mutation with respect to the length of the latent period

and the burst size, although any effect was small (Figure 23 and Table 3). This is consistent with the previous finding that the B. pertussis recA gene increased DNA synthesis most strongly in a phage uvsX- infection.

The Dependence Of The Multiplicity Reactivation Pathway On Gene 42

The potential for the existence of a direct molecular coupling between the replication and recombination systems in phage T4 has been addressed above. Formosa and Alberts (37) reported that gp42 is retained on a gpuvsX affinity column. This suggests that gpuvsX and gp42 may interact physically in vivo. Gene 42 is a dCMP-hydroxymethyase that is part of the terminal nucleotide complex associated with the replisome that acts at the replication fork. The gene 42 product has been previously reported to be required for PRRR, however it had never been tested for a role in multiplicity reactivation (MR), the other major recombinational repair pathway of phage T4 (8). As with PRRR both the uvsX and uvsY gene products have been shown by several investigators to be required for MR (8). To ascertain whether or not the gp42 protein is involved in the MR pathway, MR experiments using a phage carrying a temperature sensitive mutation in gene 42 were performed. As the data in Figure 24 clearly show, the MR pathway is dependent on a fully functional gp42. This data, along with the previous genetic analysis of the PRRR and MR pathways and the biochemical evidence coupling gp42 and gpuvsX, suggest that these two proteins may play a role in the integration of phage T4's replication and

recombination machines.

Hypothesis For The Coevolution of Replication And Recombination

The Model

This study suggests that there is an evolutionarily conserved relationship between replication and recombination. As discussed in the Introduction, recombination-dependent DNA replication has been described in a variety of organisms and appears to involve the homologous pairing and strand insertion reactions, ie. "D loop" formation of recombination to provide origins of replication that are not sequence specific. If a function, such as recombination, does in fact have the same application in several organisms, one would expect that the molecules performing that function might be at least partially interchangeable between similar pathways in those organisms. This is certainly the case for the recA genes among different bacterial species, where functional complementation in DNA recombination and repair pathways has served as the primary test of genetic identity. The same logic was applied to the complementation of recombination-dependent, ie. "recA" function dependent, DNA replication in phage T4 by the three bacterial recA genes tested. Since all three bacterial recA genes were shown to complement the uvsX gene at least partially with respect to Mode 2 DNA synthesis (Figures 5-9) the argument can be made that they each may have a similar role to the uvsX gene in the DNA synthesis in

each organisms. Evidence for this has previously been reported for E. coli. In this organism, an alternate mode of DNA synthesis initiation and the reinitiation of DNA synthesis following replication fork blockage by UV induced DNA damage have been shown to be recA dependant (67, 69, 76, 152).

The question then arises as to why these three bacterial recA proteins, each presumably generating the same recombinational intermediates as gpuvsX, don't fully complement the uvsX mutation in all pathways examined? To address this question one must first look at what the organism is accomplishing by each pathway. For example, to effect recombination in phage T4 requires the interaction of at least seven gene products, including gpuvsX, working together as a "molecular machine". Similarly, the "replication machine" consists of at least another eleven gene products working together at the replication fork (85). Within these "molecular machines" each protein is working in conjunction with every other one so that a mutation in any of them may severely impair, if not completely eliminate, the entire function. Each "molecular machine" probably has been optimized, by selection, with respect to its primary function and any deviation from the optimum configuration may result in a decrease in that function. Therefore, when a pathway such as recombination-dependent DNA replication or postreplication recombinational repair is examined, what one is observing is the interaction of two independent "molecular machines", each having been optimized for a separate function yet effectively coupled to produce a new integrated

pathway. Consequently, the final pathway is a product of selection working both within each "machine" and between "machines" to achieve the optimal function.

It is reasonable to suppose that during the evolution of an integrated pathway, specific mutations were selected for in each member "machine" that improved the integration of the two pathways. However in so doing, such a mutation might reduce the efficiency of the machine in which the interaction occurred so that another mutation might be selected which restored maximum efficiency while maintaining the increased integration brought about by the first mutation. Furthermore, it is plausible to envision a series of such mutation/selection cycles resulting in the evolution of specific coadaption of the machines. This coadaption results from the simultaneous selection within and between the "machines" towards an integrated pathway. Such a process would fit the definition of coevolution which requires reciprocity in that each of the two traits is evolving in response to the other, and specificity in that each trait is evolving in response to selection pressure directed against it individually (41). Although not ordinarily considered in a molecular context, the process of coevolution offers a reasonable mechanism by which the high level of integration of molecular pathways suggested by this study may have been attained.

Application Of The Model To The RecA Function

As a single gene product, such as the RecA protein, becomes more integrated into various cellular pathways, the selection pressure would be to maintain its function, in this case the homologous pairing and strand insertion activity, while augmenting its ability to undergo effective protein-protein interaction. The complementation experiments performed in this study asked not only if the various "recA" proteins performed the same enzymatic function as either gprecA or gpuvsX within a pathway but additionally how well they interacted with the other proteins involved. Since each of these proteins has been identified as being a "recA" protein, the question of enzymatic homology was somewhat axiomatic while the question of "machine integration" was open to examination.

Interperting The Data Under The Molecular Coevolution Hypothesis

In the experiments in which complementation was measured with respect to phage recombination (Table 2), the results followed evolutionary lines. The A. caviae (family Vibrio) is more closely related to E. coli (family Enterobacter) than B. pertussis (a distinct Genus). The A. caviae recA was able to complement the E. coli recA mutation to a relatively large extent, implying homology both at the level of protein function and at the level of interaction with the recombinational machinery. However, the B.

pertussis recA$^+$ gene could do no better than the gpuvsX in complementing the E. coli recA$^-$ mutant. The B. pertussis recA protein and the phage T4 uvsX$^+$ protein have been shown to carry out similar biochemical reaction to the E. coli recA protein (74, 154). Therefore their inability to complement the E. coli recA$^-$ mutant suggests an inability to integrate properly with the E. coli recombinational machinery.

In a similar fashion, the gpuvsX complementation results are best understood in the context of the evolution of phage T4. As a pathogen of E. coli, the phage systems evolved under the selection pressure of competition between it and its host. For example, a phage whose replication "machine" was unable to differentiate between a recombinational origin in a phage chromosome, generated by gpuvsX, and one on a remaining fragment of the E. coli chromosome, generated by gprecA, would be less fit than one that could. In this context the "interference" with gprecA function by the gpuvsX and gpuvsY protein reported by Minagawa et al. (96) is anticipated since both have evolved to perform the same function for mutually exclusive objectives, phage survival and bacterial survival. Since E. coli and A. caviae are somewhat related, the recA proteins from these two bacteria would be expected to complement the phage uvsX$^-$ mutant in a similar fashion. This is what was observed. However, a significant difference between the activity of the E. coli and A. caviae recA proteins was evident in the manner in which they complmented the uvsY$^-$ mutant (Figures 11 and 12).

Phage T4 is not in competition with Bordetella, as it is with E. coli and the interpretation of experimental results must differ accordingly. In the phage DNA synthesis experiments, Figure 9, the B. pertussis recA protein was able to complement the phage uvsX- mutant, and restore DNA synthesis to a level greater than that of the wild type T4D control. In this experiment the conditions were optimized for recombination by the use of a high multiplicity of infection ensuring the presence of at least two phage chromosomes in each cell. Under these conditions, the B. pertussis RecA protein was apparently able to catalyze the formation of multiple "D loops" and present them to the phage replication "machine" in a usable manner with an efficiency greater than wild type gpuvsX.

Molecular Coevolution And The Regulation of Mode 2 Synthesis

It is well understood that DNA replication is a highly regulated process within every organism and that its primary regulation involves control of the initiation event at designated origins (12). With the apparent ability of the recombination "machine" to generate usable replication origins at any point within a chromosome, the "origin based" method of DNA replication regulation is bypassed during recombination-dependent DNA synthesis. Never the less, the need for effective regulation of DNA synthesis presumably remains. The regulatory mechanism by which "runaway" DNA synthesis is prevented from recombinational origins is not understood. The DNA synthesis data presented in

Figures 6 and 9 suggest that in phage T4, the Mode 2 DNA replication pathway is down-regulated, achieving a normal synthesis rate in the wild type phage apparently well below the maximum attainable rate. Furthermore, this regulation seems to involve gpuvsX. The evidence that gpuvsX is involved in down regulation comes from the observation that when the B. pertussis RecA protein substitutes for gpuvsX, DNA synthesis is substantially greater than in wild type. Coevolution of the phage replication and recombination systems did not occur in the presence of any competitive exclusion between the B. pertussis RecA and gpuvsX/gpuvsY proteins. Therefore, the phage replication system is free to utilize all possible recombination origins generated by the B. pertussis recA protein. Unregulated DNA synthesis is the result.

Unlike the initial conditions of the Mode 2 DNA synthesis assay, the opportunity for phage recombination is not optimized in the PRRR experiments. In the PRRR assay only one phage chromosome is initially in the cell and DNA replication is blocked due to DNA damages introduced by UV irradiation. Under these conditions, the B. pertussis RecA protein is unable to complement the uvsX$^-$ mutation (Figures 14 - 18). Since the B. pertussis RecA protein complemented the same uvsX$^-$ mutant in the same host in the Mode 2 DNA synthesis experiments, the reason for its inability to do so in the PRRR assay must lie in the conditions of the experiment. The conditions of the PRRR assay may approximate the maximum selection pressure on the phage for the integration of the recombination and

replication machines. Under these conditions, while enzymatically the equivalent of gpuvsX, the B. pertussis RecA protein may not be able to substitute for the protein-protein interaction(s) that gpuvsX participates in within the intergrated framework of post replication recombinational repair. Thus, on the one hand, the results of complmentation of a uvsX- mutant by the B. pertussis RecA protein resulted in "runaway" replication when recombination substrates were relatively plentiful and there was no extrinsic DNA damage to interfer with DNA synthesis. But in a highly selective environment for both replication and recombinational repair, the same protein could not subsitute for the wild type phage protein. These results are taken as further evidence for the possibility of molecular coevolution of the replication and recombination machines of phage T4.

Molecular Coevolution In Higher Organisims

The final question to be addressed is whether any evidence for the molecular coevolution of replication and recombination exists in higher organisms. As discussed in the Introduction, current models of meiotic recombination, in order to account for both the biochemical and genetic data, invoke one or two rounds of DNA synthesis, depending on the model, that are primed from recombinational origins. Given that the eukaryotic "machines" for both functions are at least as complex as those of phage T4 and the universal need for strict control of DNA synthesis, the analogy

seems complete. Consistent with the hypothesis of molecular coevolution is the finding of multiple "recA" proteins from the same eukaryotic organism. Hotta and Stern (55) reported the isolation of different proteins with "recA" activity from somatic and gametic tissue in both the mouse and lily. Subsequently, a third "recA" protein was identified from the mouse (Stern, personal communication). These findings are most simply interpreted as the result of several coevolutionary processes where the same recombination mechanism, marked by the presence of the "recA" activity, has been modified to interact with different metabolic pathways within the organism.

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