RESEARCH AIMS

The mechanism by which cells recognize and complete replicated regions at their precise doubling point must be remarkably efficient, occurring thousands of times per cell division along the chromosomes of humans, and is likely to be a fundamental step required for genomic stability in all cells. Yet to date, the question of how this fundamental process occurs has not been characterized, in any cell type. Therefore, in this proposal, we will determine how replication is completed in the model organism of *Escherichia coli* and identify whether similar mechanisms operate in eukaryotes. We present preliminary evidence that the completion in *E. coli* involves an enzymatic system that effectively counts pairs and limits cellular replication to its doubling point by allowing converging replication forks to transiently pass each other before the excess, over-replicated regions are incised, resected, and joined. Completion requires RecBCD and involves several proteins associated with repairing double-strand breaks including, ExoI, SbcDC and RecG. However, unlike double-strand break repair, completion occurs independently of homologous recombination and RecA. In some bacterial viruses, we show that the completion mechanism is specifically targeted for inactivation in order to allow over-replication to occur during lytic replication. These preliminary results strongly imply that a primary cause of genomic instabilities in many double strand break repair mutants arises from an impaired ability to complete replication, independent from DNA damage processing. We describe three aims that will identify the enzymatic pathway and intermediates by which the completion of replication occurs in both prokaryotic and eukaryotic cells.

Aim A. To identify the mechanism by which replication completion occurs on the chromosome and determine which genes are required for the process to occur.

We hypothesize that the mechanism to sense and complete replication at the precise doubling point will involve converging forks transiently bypassing each other before the third unpaired (or over-replicated) sequence is resected and joined. We present preliminary evidence strongly supporting a mechanism involving transient over-replication, and have identified several intermediates arising on the chromosome. We proposed to examine mutants impaired for completion, both alone and in combination, to determine which intermediates accumulate and identify the progressive steps associated with this fundamental reaction on the chromosome.

Aim B. To determine the structural properties of converging replication forks during the replication completion process and identify the progressive intermediates associated with the reaction.

We hypothesize that converging replication forks will contain unique structural intermediates at the sites where replication is completed. Using a combination of 1-dimensional and 2-dimensional agarose gel analysis, we will identify the structural properties at completion sites *in vivo*. The analysis will be performed on defined completion sites engineered into minichromosome vectors that are optimized for this analysis.

Aim C. To determine how completion occurs in eukayotic cells, using *Saccharomyces cerevisiae* as a model.

In eukaryotes such as *S. cerevisiae*, the mechanism by which cells recognize and complete replicated regions at their precise doubling point must be remarkably efficient, occurring hundreds of times per cell division along their chromosomes. Clear homologs of *E. coli* proteins associated with completion process exist in these eukaryotes. We will use a combination of growth and plasmids stability assays, along with genomic profiling to compare the regions where replication completes in wild type and mutants of Mre11, Rad50, Xrs2, Sae2 (homologs of SbcDC and ExoI in *E.coli*), and others to determine if they are impaired in their ability to complete replication and characterize how the completion reaction occurs in these cells.

BACKGROUND AND SIGNIFICANCE

During chromosomal replication, cells tightly regulate the processes of initiation, elongation, and completion to ensure that each daughter cell inherits an identical copy of the genetic information. While the mechanisms regulating initiation and elongation have been well characterized (reviewed in (1, 2)), the process of how cells recognize replicated regions and complete replication at the precise doubling point remains a fundamental question yet to be addressed. Whether this event occurs once per generation as in *E. coli* or thousands of times per generation as in human cells, the failure to efficiently carry out this function would be expected to result in a loss of genomic stability. Considering the large number of proteins that cells devote to ensuring the fidelity of replication initiation and elongation, it seems highly probable that the final critical step in this process will be also be tightly regulated and controlled enzymatically.

In some aspects, one could argue that the efficiency of completion is likely to be more critical to the faithful duplication of the genome than that of initiation. When replication origins fail to initiate efficiently, elongation of replication forks from neighboring origins is often able to compensate (3, 4), and both prokaryotic and eukaryotic cells are able to tolerate variations in their origin number without severe phenotypic consequences (5-7). However, a failure to accurately limit or join even a single event where forks converge would be expected to result in duplications, deletions, rearrangements or a loss of viability depending upon how the DNA ends are resolved at segregation.

A number of studies suggest that an ability to sense when all sequences in the genome have doubled is critical to genomic replication. *In vitro,* converging replisomes continue through their meeting point as one replisome displaces the other, resulting in an over-replicated, third copy of the region where the forks meet (8). Complicating the process of genomic doubling even further, several studies have suggested that illegitimate initiations of replication frequently occur at single strand nicks, gaps, D-loops, and R-loops throughout the genomes of both prokaryotes and eukaryotes (9-14). Similar to when replication forks continue through a previously replicated template, each of these events would generate a third copy of the chromosomal region where the event occurs. Thus, over-replication may be inherent and promiscuous during the duplication of genomes. If true, then to ensure that each sequence of the genome replicates once, and only once per generation, cells must encode an enzymatic system that is essentially able to count in pairs, and efficiently degrade odd or over-replicated regions until the two nascent end pairs of replication events can be joined.

The model organism *E. coli* is particularly well-suited to dissect how this fundamental process occurs. In *E. coli*, the completion of replication occurs at a defined region on the genome, opposite to the bidirectional origin of replication (15). Most completion events can be further localized to one of six termination (*ter*) sequences within the 400-kb terminus region due to the action of Tus, which binds to *ter* and inhibits replication fork progression in an orientation-dependent manner, in effect stalling the replication fork at this site until the second arrives (16, 17). Although Tus confines converging replication forks to a specific region, it does not appear to be directly involved in the completion reaction since *tus* mutants have no phenotype and complete replication normally (18). Furthermore, plasmids and bacteriophage lacking *ter* sequences are maintained stably (19).

PRELIMINARY DATA

recBC and *recD* mutants exhibit phenotypes that suggest a fundamental role in DNA replication

Many mutants impaired for either replication initiation or elongation were initially isolated based on their growth defects or an impaired ability to maintain plasmids (20-22). We

reasoned that mutants impaired for the ability to complete replication might be expected to exhibit similar phenotypes and initially focused our attention on the properties of *recBC* and *recD* mutants. RecB-C-D forms a helicase-nuclease complex that is required for homologous repair of double-strand breaks in *E. coli* (23, 24). The enzyme utilizes specific DNA sequences, termed Chi sites, to initiate recombination between pairs of molecules. Loss of RecB or C inactivates the enzyme complex, whereas loss of RecD inactivates the nuclease and Chi recognition, but retains helicase activity (23, 24).

Relative to wild type, *recBC* cultures grow poorly and produce large numbers of nonviable cells, whereas *recD* cultures grow for a longer period and reach a higher cell density than wild type cultures (**Fig1A**) (25-28). By comparison, in the absence of RecA which is essential for all homologous recombination and RecBCD-mediated double strand break repair, cultures grow comparatively well, arguing that some function of RecBC is unique from homologous repair and DNA damage.

Mutations inactivating RecBC or RecD also affect the stability of plasmid, *recD* mutants rapidly lose plasmids in the absence of selection. *recBC* strains are also less able to retain plasmids relative to wild type cultures, although overall cell viability is similarly reduced (**Fig1B**). These features are again distinct from other recombination mutants, including *recA* in which plasmids replicate stably (**Fig1B**) (28, 29).

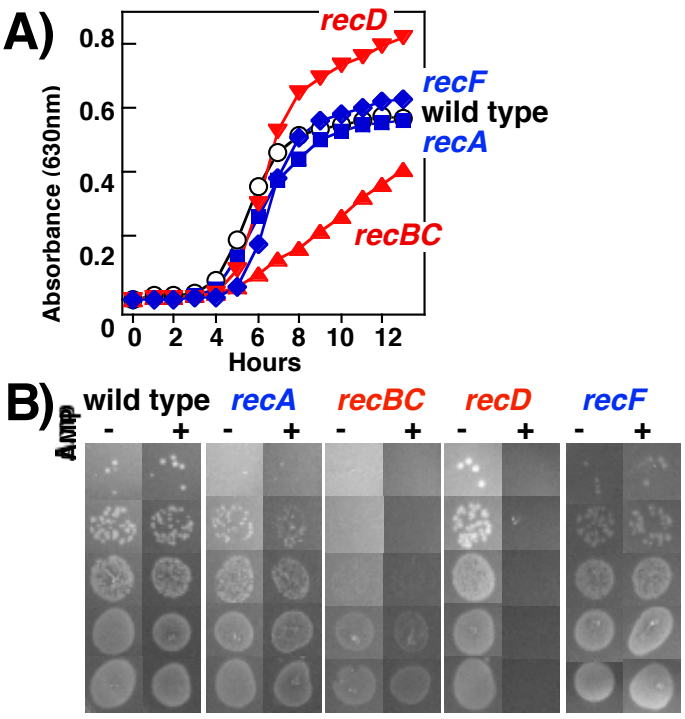

Figure 1. *recBC* and *recD* mutants exhibit growth abnormalities and an impaired ability to maintain plasmids. A) Unlike other recombination mutants, the growth of *recBC* mutants is impaired, whereas *recD* mutants grow for a longer period of time and reach a higher density relative to cultures of wild-type. The absorbance at 630nm of cultures grown at 37°C is plotted over time. B) *recBC* mutants and *recD* mutants exhibit plasmid instability. Cultures containing the plasmid pBR322 were grown for 30 generations before 10μl drops of 10fold serial dilutions were plated with and without ampicillin to determine the fraction of cells that retained the plasmid in each strain.

When we examined the form of the replicating plasmids in each of these mutants, we observed several phenotypes worth considering. Plasmids grown in *recD* mutants continue to replicate past the doubling point, producing a range of odd- and even-numbered mutlimeric circles as well as long linear-multimers (**Fig2A** and **B**). The unstable phenotype in *recBC* is distinct from *recD* mutants, and involves an elevated level of gapped molecules and dimer plasmids, rather than extensive over-replication.

Several observations argue that these phenotypes are related to replication abnormalities, rather than defects in double strand break repair. If the plasmid instability in *recBC* and *recD* mutants arose from an inability to process double strand breaks, *recBC* and *recD* would be expected to accumulate broken intermediates. Yet, the proportion of broken, linear plasmids is actually lower in *recBC* or *recD* cultures relative to wild type or other recombination mutants (**Fig2A**). Additionally, double strand breaks are estimated to occur at frequencies of 0.01-1 per

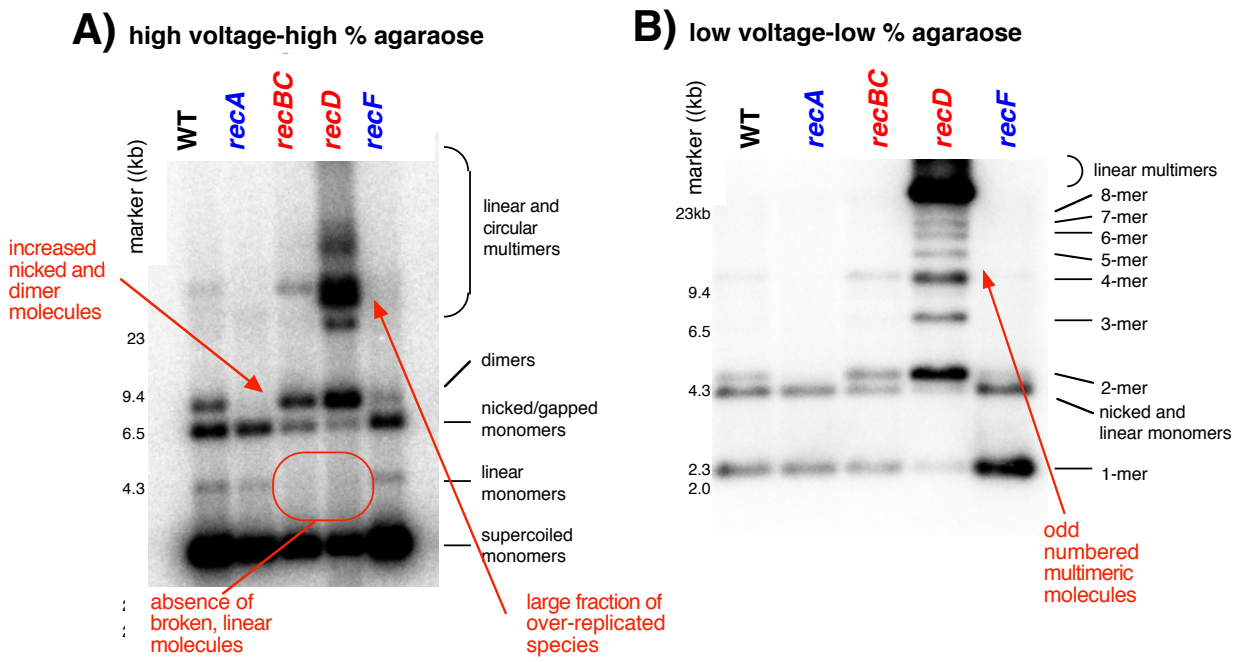

Fig 2. Unlike other recombination mutants, plasmids replicating in *recBC* cultures accumulate nicked and dimer plasmids, whereas *recD* cultures accumulate over-replicated, multimeric species. A) Linear monomers, indicative of double-strand breaks, are absent in both *recBC* and *recD* mutants relative to wild-type cultures. Total genomic and plasmid DNA was prepared from replicating cultures containing pBR322 and examined by Southern analysis using 32P-labelled pBR322 as a probe. DNA was electrophoresed through a 1.0% agarose gel in TAE at 4 V/cm B) Unlike other recombination mutants, replication of plasmids in *recD* mutants leads to multimeric circles that contain both odd and even numbers of plasmid copies. Samples were analyzed as in (A) except the DNA was electrophoresed through a 0.5% agarose gel in TAE at 1V/cm. Resolution under these conditions resolves molecules primarily based on the molecule's size and reduces the impact that shape has on the migration rate of the molecule.

4.5-Mb of replicated genome (30), making it unlikely to account for instability on the 4.5-kb plasmid. Finally, plasmids are hyper-stable and replicate normally in *recA* mutants that are defective in homologous recombination and double strand break repair (**Fig2**). Taken together, these observations argue strongly against the idea that the growth and plasmid abnormalities in *recBC* and *recD* mutants arise from defective processing of double strand breaks. However, these phenotypes would all be consistent with those expected of mutants impaired in their ability to recognize and complete replication at the doubling point.

Other phenotypes associated with *recBC* mutants also suggest the gene products play a role in completing replication on the chromosome. Following UV-irradiation, many hypersensitive recombination mutants, including *recA*, cease replicating immediately after replication encounters the DNA damage (31, 32). However, *recBC* mutants are unusual in that they initially recover and continue to replicate similar to wild-type cells. The replication continues normally for a short period before DNA synthesis ceases ((31) and **Fig3**), indicating that the defect in *recBC* mutants is distinct from RecA and arises at the final stages of replication. Consistent with this interpretation, *ter* sequences are hot spots for RecBCD-

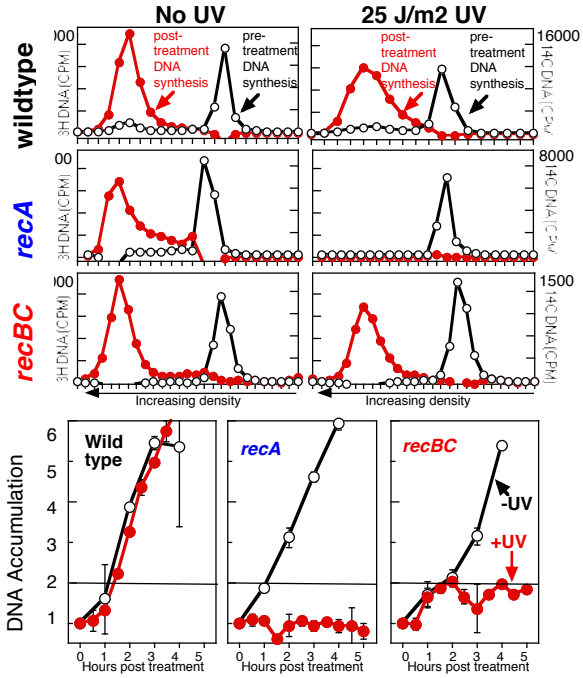

Fig 3. Following UV-irradiation, *recBC* mutants initially recover replication, but then replication arrests after an approximate doubling of their genomic material. **A.)** *recBC* mutants initially recover replication. [14C]thymine prelabeled cultures were irradiated or mock treated, and resuspended in media containing [3H]5- bromodeoxyuridine for 1 hour to density label the replication occurring during this period. The denser replicated DNA is then separated in alkaline CsCl density gradients and quantified. Both wild type and *recBC* mutants restore replication equally well during the 1st hour after UV treatment. *recA* mutants do not recover. **B.)** Replication arrests in *recBC* mutants after an approximate doubling. Cultures growing in 3H-thymine were irradiated or mock treated and sampled at various times to determine the total amount of [3H]DNA accumulated. Wild type cells recover replication and continue to grow following irradiation. *recA* mutants do not recover replication. *recBC* initially recover replication, but then replication arrests once the DNA has approximately doubled.

mediated recombination (33, 34), implying that the region where replication completes contains substrates frequently recognized by RecBCD *in vivo*.

To directly examine whether RecBCD functions in completing replication on the chromosome, we profiled the genomes of replicating wild-type and mutant cultures using high-throughput sequencing. In replicating wild-type cultures, the copy number of sequences is highest surrounding the bidirectional origin, then gradually decreases until it reaches the terminus where replication completes (**Fig4A**). In mutants lacking RecBC, there is a marked decrease in the copy number of sequences specifically in the terminus region. The terminus sequences in *recBC* mutants are under-represented by up to two fold, relative to wild type cultures. Assuming that greater than half of the sequence reads correspond to parental DNA, one can infer that the majority of cells in the population have difficulty replicating or maintaining sequences in this region. Conversely, an increase in the copy number of sequences within the terminus region is observed in *recD* mutants, which inactivates the exonuclease activity of the enzyme complex (**Fig4B**). Consistent with the observations on plasmids, the results indicate that the RecBCD complex is required to allow the efficient and accurate completion of replication on the chromosome. The presence of the over-replicated intermediate inside the boundary of the *ter* sites in *recD* mutants implies that converging forks transiently pass each other before the nuclease activity of RecBCD resects these over-replicated intermediates back to the doubling point. The lack of sequences at the termination region in *recBC* mutants reveals that the enzyme complex is required to resolve and join the convergent forks at the doubling point. In its absence, the DNA ends of the converging forks remain subject to nucleolytic attack and are degraded.

Importantly, the completion of replication on the chromosome occurs normally in *recF* and *recA* mutants, indicating that the completion reaction catalyzed by RecBCD does not require homologous recombination or involve the repair of double-strand breaks (**Fig4B**). We are not aware of any recombination models for repairing collapsed forks that do not involve RecA, nor do any known recombinational processes require RecBC, but not RecA. Thus, the lack of the terminus region DNA in *recBC* mutants is inconsistent with the idea that the intermediates are

associated with a recombination defect or collapsed replication forks occurring in this region. We infer that the impaired ability to complete replication in *recBC* mutants is independent from its role in double-strand break repair, and likely accounts for the poor growth of these cells relative to *recA* or other recombination mutants.

Additional genes associated with double-strand break repair are also involved in completion. SbcDC, a structure-specific helicase-nuclease, and ExoI, a prominent 3'-5' exonuclease, suppress the growth defects of *recBC* mutants when mutated, and lead to plasmid instability similar to *recD* (25, 35, 36). Mutations in eukaryotic homologs of these proteins are associated with genetic instabilities and impaired double-strand break repair (37). In replicating *sbcDC xonA* mutants, a similar over-replication of the terminus region is observed (**Fig4C**), indicating that these genes play a role in processing or resolving the transient over-replicated regions.

A recent study has shown that mutants lacking RecG, a translocase important for dissolving mis-primed events after DNA damage, also over-replicates its terminus region (**Fig4C** and (38)). In both *recD* and *recG* mutants, the DNA ends from unresolved completion events lead to over-replication that can also be observed on plasmids. However, the over-replication that occurs in each of these mutants is

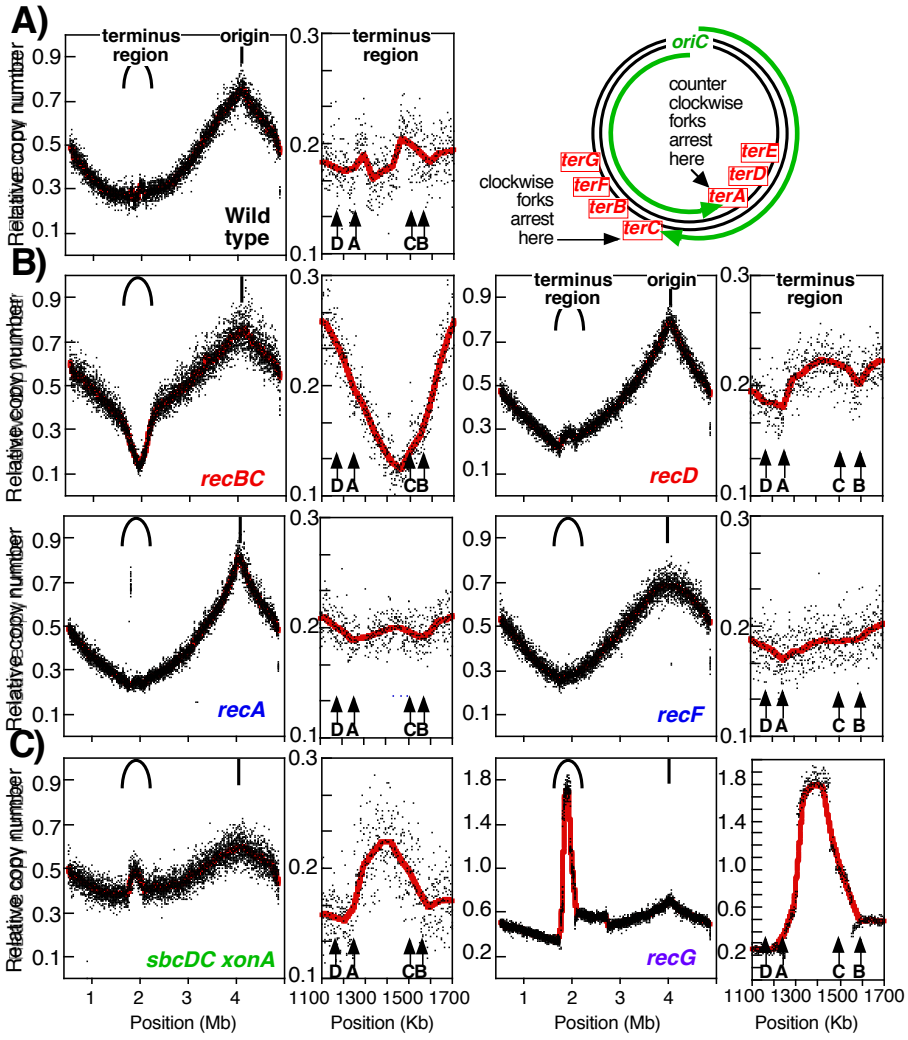

Figure 4. RecBCD resolves and completes replication at the doubling point on the chromosome, independent of homologous recombination. A) In wild-type cultures, replication proceeds bidirectionally from the origin and completes in the terminus region. Genomic DNA from replicating cultures was purified, fragmented, and profiled using high-throughput sequencing. Sequence read frequencies, normalized to stationary phase cells, are plotted relative to their position on the genome. The terminus region of the chromosome, containing *terD*, *A*, *C*, and *B*, is shown next to each plot. An 8kb floating average of the sequence frequency is plotted in red. B) *recBC* mutants fail to complete replication, leading to degradation of the terminus region. *recD* mutants fail to resect and limit replication to the doubling point, leading to over-replicated regions in the terminus. Completion occurs normally in *recF* and *recA* mutants. C) Over-replicated regions persist in *sbcDC xonA* mutants. Illegitimate re-initiations of replication occur in *recG*. Note the different scale for *recG*.

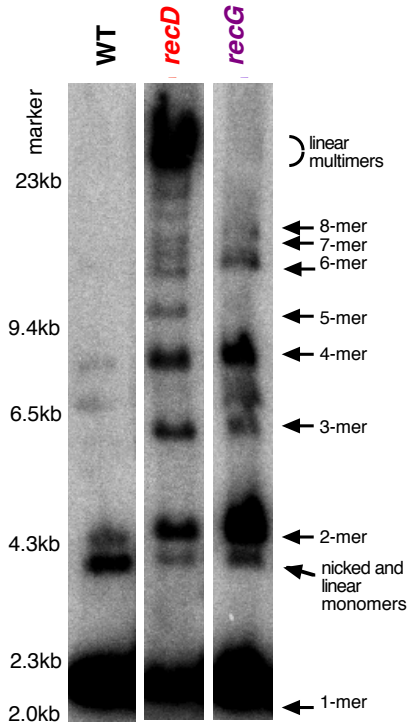

Figure 5. Impaired completion events lead to distinct intermediates on plasmids in *recD* and *recG* mutants. *recD* mutants accumulate long linear multimers, as well as both odd- and even-numbered multimeric circles. In *recG* mutants, the over-replicated products consist of predominantly even-numbered, multimeric circles and lack any linear multimers. DNA was analyzed as in Fig2B.

distinct in several aspects. The aberrant long linear-multimeric intermediates that accumulate in *recD* mutants do not appear in *recG* mutants. In addition *recD* mutants are unique in that they contain prominent odd numbered circular plasmid multimers, suggesting that RecD contributes to efficient pair recognition prior to resolution. In contrast, over-replicated plasmid species in *recG* mutants predominantly consist of even-numbered circular multimers (**Fig5**), suggesting that these mutants retain the ability to recognize and resolve molecules as pairs. We interpret these results to suggest that although RecG plays a role in preventing illegitimate re-initiations from occurring, it is not directly involved in recognition or joining of the linear DNA ends at the doubling point. Consistent with this interpretation, *recG* mutants grow normally and plasmids are stably maintained (data not shown). *recG* mutants are also constitutively induced for SOS expression (39), which may contribute to the over-replication that occurs on plasmids and the chromosome in these strains.

Many lytic viruses, including bacteriophage lambda, have two modes of replication, an early phase in which its genome doubles similar to the bacterial chromosome, and a late phase in which the viral genome is amplified before packaging and release from the cell (40). Late phase replication in phage lambda requires expression of the phage Gam protein, which targets and inactivates RecD and SbcDC in the host (41, 42). Similar to the amplification of phage and plasmid DNA (36), we observed that *gam* expression results in an over-replication of the terminus region (**Fig 6**). Thus, to initiate genomic amplification during lytic infection, the phage targets and inactivates the cellular mechanism that limits replication to the doubling point, allowing over-replication to occur.

Taken together, the plasmid and chromosomal data presented here indicate that RecBCD is directly involved in limiting replication events and resolving them at points where sequences have doubled. This process is distinct from double-strand break repair and occurs efficiently in the

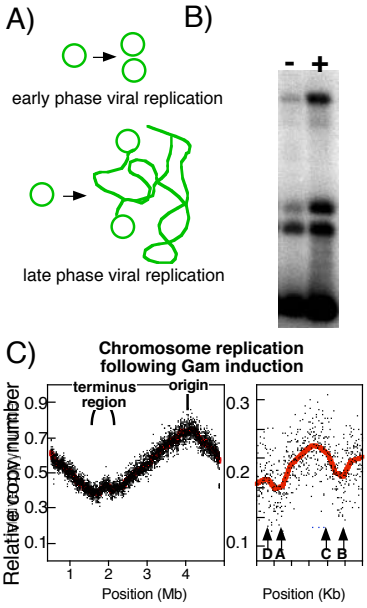

Figure 6. Induction of the bacteriophage *gam* gene inactivates the cellular mechanism that limits replication to the doubling point. A) Lambda late phase replication requires *gam* induction. B) *gam* induction leads to over replication on plasmids and C) the chromosome. Cells containing an arabinose-inducible *gam* gene were grown with arabinose and prepared as before.

absence of RecA or homologous recombination on both plasmids and the chromosome. However, when one considers the mechanism by which double strand breaks are repaired, it becomes clear how these two processes may be related (**Fig 7**). Double-strand break repair in *E. coli* requires both RecA and RecBCD function. RecA is believed to pair the severed strands with intact homologous duplex DNA (23, 24). Once this occurs, the sequences between the opposing strands are replicated and joined using the second molecule as a template. A structurally similar process must also occur whenever two replication forks converge. However, in the case of completion, the opposing nascent strands have been brought together by replication forks and should be independent of RecA.

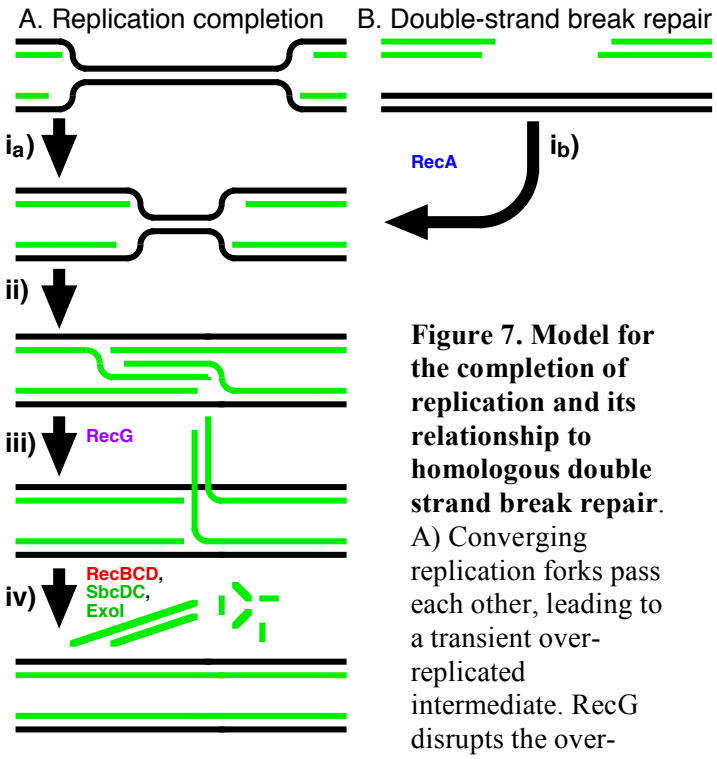

Figure 7. Model for the completion of replication and its relationship to homologous double strand break repair. A) Converging replication forks pass each other, leading to a transient over-replicated intermediate. RecG disrupts the over-replicated intermediate, preventing re-initiation and illegitimate replication. RecBCD, Exo I, and SbcDC are involved in unwinding and resolving the over-replicated regions at the doubling point. Other intermediates and gene products involved in this reaction remain to be identified. B) RecA initiates homologous double-strand break repair by pairing DNA ends with a homologous double-stranded template, generating an intermediate that can be repaired by completing the replication of the intervening sequences.

During double strand break repair, RecBCD is proposed to process the DNA ends prior to strand invasion. In most models, this processing is restricted to the early stages of the reaction (23, 24). However, *in vivo* experiments have suggested that strand invasion can occur in the absence of RecBCD, but that its function is still required if viable recombinants are to be recovered (43, 44), arguing that RecBCD enzyme function acts late in the recombination process, perhaps by actively resolving the re-replicated regions at the doubling point.

Considering the chromosomal phenotypes of *recBC* and *recD* mutants, it is tempting to speculate that monomeric linear plasmid species, which are diminished or absent in these strains, represents an incised intermediate of over-replicated products (**Fig2A**). However, the precise substrates that occur during the completion process, as well as the other genes involved in processing and recognition remains to be determined, and is the primary objective of aims 1 and 2.

Mutations in several eukayotic and mammalian double strand break repair genes also exhibit growth defects and genetic instabilities in the absence of exogenous DNA damage. Many of these have clear homologs to the proteins we have thus far identified in *E. coli*. Whereas a single termination event occurs per cell cycle in *E. coli*, completion must occur hundreds of times per cell division in yeast and thousands of times per division in humans. Considering these results, it seems highly likely that a homologous system will operate to ensure efficient and accurate completion events occur in eukaryotic cells. Identifying this mechanism and the genes involved is the primary goal of Aim 3.

RESEARCH PLAN OF ATTACK

A. To identify the mechanism by which replication completion occurs on the chromosome and determine which genes are required for the process to occur.

The preliminary data strongly supports the hypothesis that the completion of DNA replication involves an enzymatic system that effectively counts pairs and limits cellular replication to its doubling point by allowing converging replication forks to transiently pass each other before the excess, over-replicated regions are incised, resected, and joined through the combined action of RecBCD, and ExoI, SbcDC and RecG. Remarkably, we demonstrate that the completion reaction is distinct from, and occurs independently of homologous recombination, double-strand break repair, and RecA. Our current working model, presented in **Fig 7**, could explain the relationship that double strand break repair has with the completion of DNA replication. However, as shown, several steps of this fundamental process in genome duplication remain undefined. And the model also makes a number of predictions as to how double strand break repair occurs *in vivo*. The experiments proposed in this aim are designed analyze the chromosome to identify additional genes associated with this reaction, differentiate whether over-replication is an enzymatic or passive process, and determine the method by which over-replicated sequences are removed and resolved.

Are other genes products involved in the reaction?

Although our experimental rationale initially led us to focus on the *recBC* and *recD* mutants, it is likely that additional genes will participate in this reaction. **Table 1** includes an initial list of candidate genes products and their potential roles, which we will examine for abnormalities in the terminus region using DNA-seq and genomic profiling as shown in **Fig 4**. Additional genes will be examined as suggested by intermediates identified aim 2. The size of the *E. coli* genome allows us to comfortably sequence 30 strains per lane and obtain enough reads for accurate profiling. Although we expect many of these gene products to exhibit abnormal phenotypes, even negative results are useful in defining the specificity of this pathway and how completion occurs.

TABLE 1. CANDIDATE GENES TO BE EXAMINED FOR IMPAIRED COMPLETION.

CANDIDATE GENE(S)	PHENOTYPES	RATIONALE/POTENTIAL ROLE
recN	hypersensitive to double strand breaks. The protein binds DNA ends, and is often discussed as a functional homolog of Ku.	Potential protection, of over-replicated free ends. Hairpin forming activity? (steps iii and iv).
radA	X-ray sensitive mutant that encodes a poorly characterized RecA paralog.	Potential strand exchange protein specific for the completion reaction, allowing fork bypass (step ii) or resolution at the doubling point (step iv).
recJ ***xse*** ***xth***	These are other exonucleases with roles in replication, repair, and recombination.	The genes, particularly RecJ may be involved in allowing over-replicated intermediates to persist, and processing ends at resolution (step iv).
ruvAB ***ruvC*** ***rusA***	Mutants are hypersensitive to DNA damage. The proteins are associated with branch migration and resolution of DNA junctions.	Potential completion specific role in allowing fork bypass (step ii) or resolution at the doubling point (step iv).
polB, ***dinB*** ***umuCD***	Alternative DNA polymerases that have been shown to replicate through specific DNA lesions and difficult DNA structures.	A potential role in allowing forks to bypass (step ii) or filling in gaps at resolution (step iv).
recQ ***uvrD*** ***rep*** ***dinG***	These are specialized DNA helicases that have been shown to have roles in replication and repair.	Potential completion specific role in allowing fork bypass (step ii) or resolution at the doubling point (step iv).

Is over-replication passive or does it require enzymes?

The presence of the over-replicated intermediates on the chromosome is a compelling argument that the completion reaction is enzymatically regulated. However, several questions remain about the mechanism by which completion occurs. One important question centers on whether converging replication forks bypass each other passively, with one fork simply plowing off the other? Or are enzymes required to catalyze template switching and allow bypass and over-replication to occur?

The absence of DNA in the terminus region of *recBC* mutants could reflect either 1) that RecBC is required to allow replication forks to bypass each other, or 2) that RecBC fails to join and resolve DNA ends, leading to extensive degradation in the terminus. This can be addressed by examining the terminus region in *recBC* mutants that also lack SbcDC and Exo I. These exonucleases are known to suppress the growth defect of *recBC* mutants and lead to overreplication on plasmids (25, 35, 45). If the terminus DNA on the chromosome is restored (or over-replicated) in the suppressed *recBC* mutants, it would indicate that the RecBC enzyme is not required for fork bypass, but is required for efficient joining of the DNA ends during completion to limit the degradation by these exonucleases.

A second possibility is that RecBCD-catalyzed completion normally occurs precisely at forks collisions, without overreplication. But that RecA-catalyzed strand exchange can lead to template switching and over-replication when completion is impaired. To address this, all mutants exhibiting over-replication phenotypes, *recD recG* and *sbcDC xonA*, will be examined in a *recA* (and potentially *radA*) background to determine if enzymatic template switching is required for replication forks to bypass each other and over-replication to occur.

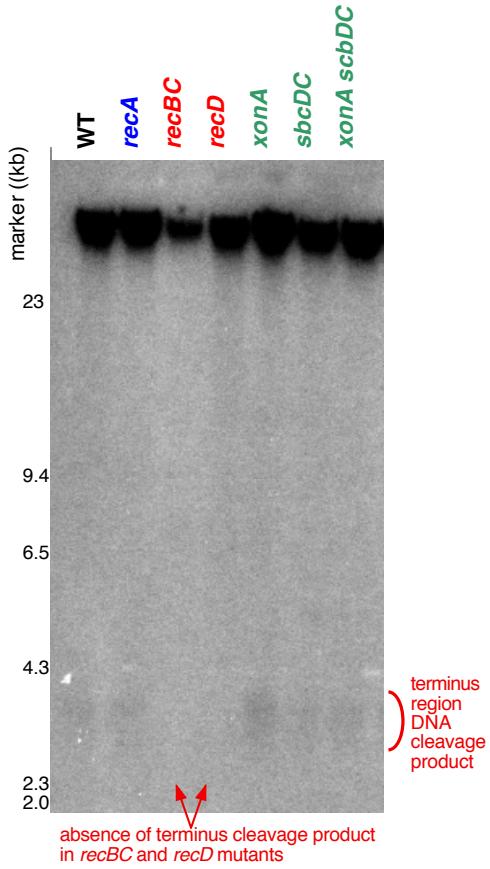

Figure 8. A cleavage product made up of terminus region DNA is detected in wild type genomic samples, but is absent in *recBC* and *recD* mutants. A southern analysis of genomic DNA purified from replicating cultures of each strain. DNA was electrophoresed through an alkali-denaturing agarose gel and probed with a 32P-labeled PCR fragment of the terminus region. While most DNA migrates as large, >23kb fragments, a small cleavage product of the terminus region DNA is detected in all strains except *recBC* and *recD*.

How are over-replicated sequences processed?

Over-replicated intermediates are observed on the chromosome in several mutants. Importantly, similar but less extensive intermediate signatures are also observed in wild type cells at both *terA* and *terC* junctions, suggesting that these intermediates occur as a normal part of the completion reaction (**Fig 4A**). The over-replicated sequences are likely to initially exist as DNA flaps that are attached to the chromosome. Removing these sequences could occur through several mechanisms. It may involve endonucleolytic incision of the flaps at the doubling point junction, they may processed into hairpin intermediates and resolved, or they may simply be exonucleoytically degraded back to the doubling point junction.

In wild type and mutant plasmids, we observe an intermediate that migrates similar to a linearized plasmid in all strains except *recBC* and *recD* (**Fig 2A**). We hypothesized that this may represent a cleavage or incised product following

resolution of the over-replicated intermediates.

To determine if this intermediate also occurs on the *E. coli* chromosome, we purified total genomic DNA from replicating cultures of wild type cells, as well as various mutants and electrophoresed the genomic DNA (unrestricted) through an alkaline agarose gel. In theory, all of the DNA should be high molecular weight. However, when we probed with a segment of the terminus region, we detected fragments a few kilobases in length in wild type cells and several mutants. Interestingly, and consistent with the observations of replicating plasmids, both *recBC* and *recD* lacked these intermediates (**Fig 8**). The observation is consistent with the idea that RecBCD is involved in incising the over-replicated regions, to generate these chromosomal replication intermediates during resolution.

We propose to further characterize these intermediates, by 1) using probes to determine the boundaries of the incisions regions surrounding the completion region, 2) cloning and sequencing these DNA fragments to determine whether they contain inverted repeats, or evidence of hairpin structures, 3) compare their presence in both neutral and alkali gels, and use strand specific probes to determine whether over-replicated regions contain double stranded or single stranded flaps 4) and examine whether the presence of this intermediate increases, decreases, or is altered in double mutants lacking *recBC* or *recD* as well as other genes associated with the the completion reaction.

Are the resolution-joining and pair recognition activities within RecBCD itself?

The lack of DNA in the terminus of *recBC* mutants and odd-numbered over-replicated multimers in *recD* mutants, strongly argues that the RecBCD complex is directly involved in sensing and resolving convergent forks the doubling point. RecB-C-D is a remarkably complex enzyme that contains dual helicase and translocase activities with different processivities, an ability to track and loop DNA strands, exonucleolytic activity, and endonuclytic activity at specific DNA sequences, termed Chi sites (**Fig 9** and (46-48)). Chi sites alter the helicase and nuclease activity of the complex following incision, are over represented in the *E. coli* genome, and are found predominantly on the leading strand template (49). Dr. Gerald Smith's lab has an array of point mutants that selectively inactivate each of these activites in the complex. Using these point mutants with altered activites, we will determine how each of the complex's activities affect recognition of DNA pairs, over-replication, incision, and resection. These mutants will also allow us to examine how Chi affects the resolution site of the convergent forks, both on plasmids and on the *E. coli* chromosome. The Smith lab, which identified many of these point mutations, has solved the RecBCD crystal structure (46), purified many of these mutant proteins, and characterized them biochemically (46, 50-52). The Smith lab is quite interested in our preliminary results and is anxious to examine RecBCD properties on some of the substrates suggested by our observations. Thus, in collaboration with Gerald Smith and his group, point mutants exhibiting activities of interest will be further characterized biochemically on structures identified in Aim A and B (letter of support, attached).

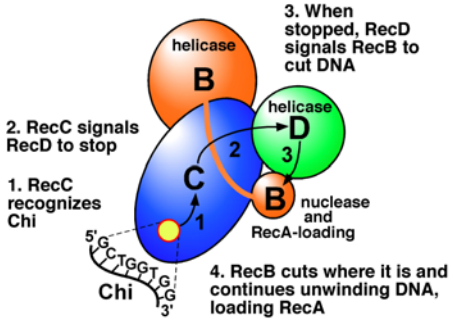

Figure 9. The RecBCD complex contains several coordinated activities. Point mutants exist that selectively inactivate each of these activities. (image courtesy of Gerald R Smith)

Aim B. To determine the structural properties of converging replication forks during the replication completion process and identify the progressive intermediates associated with the reaction.

We hypothesize that the sites where replication is completed will contain unique structural intermediates. We have experience with characterizing the structure of replicating DNA molecules using 2D agarose gels and have used this technique previously to describe the behavior of replication forks when they encounter UV-induced damage (53-56). **Fig 10** shows that the technique can be used to observe several structural intermediates on replicating plasmids of pBR322. While suggestive, the use of pBR322 also has limitations for analyzing the completion reaction. pBR322 contains a unidirectional origin and lacks replication (*ter*) barriers that would contain the completion reaction to a specific region. These features make it difficult to attribute observed structures specifically to the completion reaction. Therefore, we propose to utilize the "minichromosome" vectors shown in **Fig 11**. The vectors contain a lambda birdirectional origin and maintain a copy number of 20-30 copies/genome. pLamKanTer also contains two *ter* sequence barriers oriented similar to the chromosome to contain the reaction to the lower half of the plasmid. Restriction sites allow the origin- and terminus-containing fragments to be separated and analyzed independently by 1D and 2D gel analysis so that gaps or the overreplicated, forked-structures can be identified and characterized for their length, shape, and sequence. The use of electron microscopy and strand specific probes can be used to further identify the strandedness and structures observed (54, 55). We are confident the approach will be useful in characterizing the completion intermediates in detail.

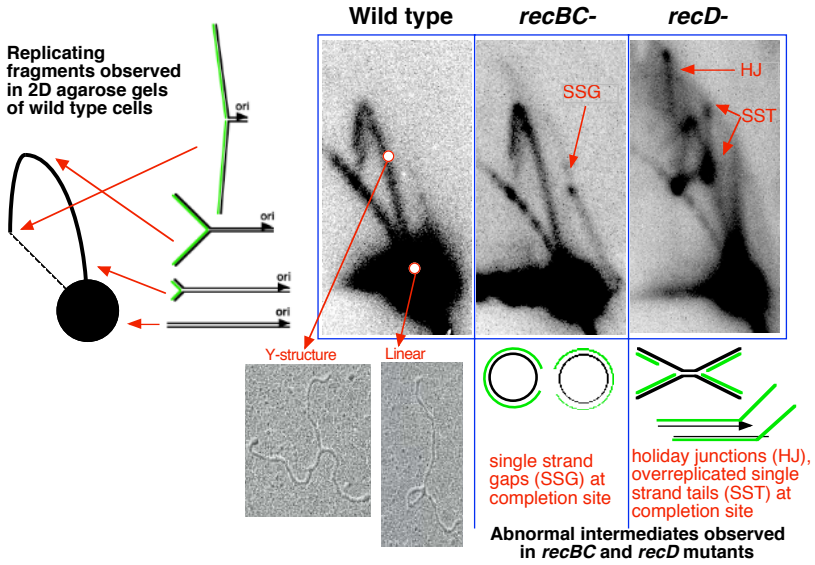

Figure 10. Abnormal intermediates observed on replicating plasmids in *recBC* and *recD* mutants. In this analysis, pBR322 plasmid is linearized at its unidirectional origin of replication and examined by 2D gels. Nonreplicating plasmids run as a linear fragment and all normal replicating fragments form Y structures as shown in the first panel. In wild type cells, only normal replicating fragments are detected. Plasmid structures can be excised from the gel and confirmed by electron microscopy.

We will initially test each mutant to identify which enzymes are required to 1) allow forks to bypass each other, 2) incise the over-replicated flaps, and 3) resect or degrade the over-replicated sequence. Once the specific intermediates are identified, the analysis can then be extended and optimized to identify these intermediates on the chromosome as described in Aim A.

Single mutants that accumulate distinct intermediates will then be tested in combination to determine the order in which the gene products and

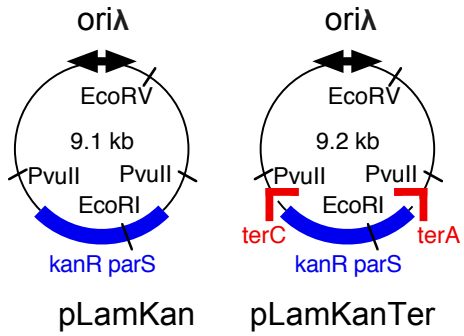

Figure 11. Minichromosome vectors for the analysis of replication completion.

intermediates occur during the completion process. As in any biochemical pathway, the intermediates associated with the gene product that operates earlier in the repair process would be predicted to accumulate in the double mutant, allowing us to order the progressive steps in the repair process.

We expect these results to allow us to identify the structures and progressive intermediates that are associated with the completion reaction.

TABLE 2. YEAST CANDIDATE GENES TO BE EXAMINED FOR IMPAIRED COMPLETION

CANDIDATE GENE	Homolog/Activity
WT	Strain BY474
rad51	Homolog of RecA
pif1	Homolog of RecD
mre11	Homolog of SbcC
rad50	Homolog of SbcD
xrs2	part of Mre11-Rad50 complex (human Nbs1)
sae2	Homolog of ExoI
rad52	Homolog of RecO
sgs1	Homolog of RecQ
exo1	Exonuclease involved in recombination
rrm3	(human ATM)
mus81	Cleaves cruciform DNA

Aim C.*To determine how completion occurs in eukaryotic cells, using S. cerevisiae as a model.*

In eukaryotes such as *S. cerevisiae*, the mechanism by which cells recognize and complete replicated regions at their precise doubling point must be remarkably efficient, occurring hundreds of times per cell division along their chromosomes. Clear homologs of *E. coli* proteins associated with completion process exist in these eukaryotes. We will use a combination of growth and plasmids stability assays, along with DNA-seq to compare the regions where replication completes in wild type and mutants to determine if they are similarly impaired in their ability to complete replication and characterize how the completion reaction occurs in these cells. An initial list of candidates to be examined is shown in TABLE 2.

Mutations in several eukaryotic double strand break repair genes also exhibit growth defects and genetic instabilities in the absence of exogenous DNA damage, suggesting that the defect in some of these mutants may relate to an impaired ability to complete replication. Mre11 (the homolog of SbcC), exhibits impaired growth in yeast and mammalian cell cultures and is embryonic lethal (57, 58). It is expressed in replicating cells and predisposes individuals to cancer when mutated (59, 60). Mutations in other gene products, Nbs1, and Rad50 (homolog of SbcD) are also associated with cancer predisposition and developmental abnormalities, even in the absence of exogenous DNA damage, and are speculated to function at replication forks (61, 62), suggestive that they may similarly have roles fundamental to the DNA replication process.

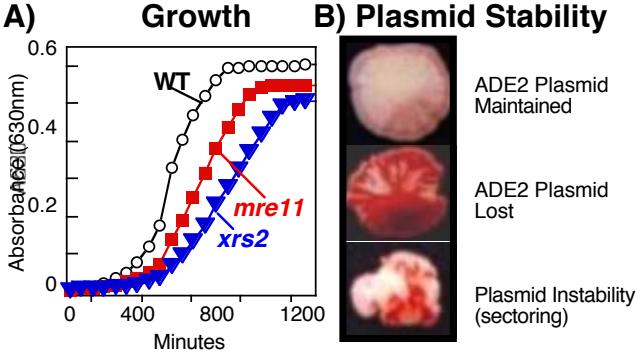

Figure 12. Assays to monitor growth and plasmid stability in yeast. A) Growth of *mre11* and *xrs2* mutants relative to wildtype is shown in YTE media at 30C. B) The loss or maintenance of plasmids expressing ADE1 can be following in *ade1* mutants. These assays will be used in combination with genomic profiling to characterize mutants for their ability to complete replication.

Growth and plasmid stability

We propose to examine the growth and plasmid stability of several candidate mutants as a starting point to characterize candidates with potential replication defects in completion, similar to our analysis in *E.coli*. Initial mutants to be examined will include the homologs of *E.coli* completion mutants, as well as others reported to operate at replication forks or associated with recombination during replication (**Table 2**). Growth curves of yeast (**Fig12A**), colony size, and cell morphology may all be informative in this

analysis. Plasmid stability in yeast can be monitored by expression ADE2 gene from plasmids in *ade2* mutants, providing a colormetric assay (**Fig12B**) (63). A similar assay was originally used to identify genes associated with replication elongation and initiation (22). Surprisingly, the comparative ability of several recombination and break repair mutants to maintain plasmids has not been directly examined, and is likely to be informative.

Direct examination of completion on the yeast chromosome

Yeast contain well-defined origins, many of which fire with greater than 50% efficiency, and a genome that is only ~3 fold larger than *E. coli*. These properties will allow us to directly examine whether over- and under-replication intermediates accumulate in these mutants at regions where replication forks meet between origins. We propose to compare the profiles of replicating genomes in wild type and mutants cultures, similar to how we have done for *E. coli* in **Fig 3**.

Yeast will be synchronized and released, and their progression through S-phase will be monitored by flow cytometry (**Fig 13**). At times throughout S-phase, the DNA will be purified, sequenced, and analyzed similar to that in **Fig 4**. To detect of over-replication at completion points, we will be looking for regions with increased copy numbers that arise between origins, in late S-phase, and appear in mutants but not wild type cultures. Mutants with under-replication phenotypes, similar to *recBC* mutants, can be identified in a similar manner. However in this case, to differentiate mutants with elongation defects from those specifically impaired for completion events, it will also be necessary to profile their progression through S-phase, to ensure that early phases of replication progress normally. Recent studies have used this approach to map origin firing through S-phase in wild type cultures (79). These studies define multiple inter-origin regions that will be suitable to characterize, and suggest some provocative regions to examine, consistent with completion, in late S-phase.

Once intermediates are identified, minichromosome plasmids could then be characterized in yeast similar to for *E. coli* in Aim 2.

As Gerald Smith mentions in his letter of support, he is interested in seeing us utilize *S. pombe* as a model for these studies and has offered to supply us with these mutants and advice. However, considering that we have already obtained most of the *S. cerevisiae* mutants and worked out many of our assays conditions, we would like to proceed with this model. However, we recognize many of Gerry's points as to advantages with *S. pombe*, and given the similarity between systems, we hope to collaborate with him and potentially proceed concurrently.

We believe the demonstrated presence of mutants impaired for completion in *E.coli*, along with the clear homologs that exist in yeast, make this aim likely to succeed. If over- and under-replication regions are detected between origins in the homologous yeast mutants, similar to *E. coli*, it would strongly support the idea that the completion process is conserved between prokaryotes and eukaryotes. And would support the idea that a primary cause of genomic instability in many double strand break repair mutants, arises from an impaired ability to complete replication, independent from DNA damage processing.

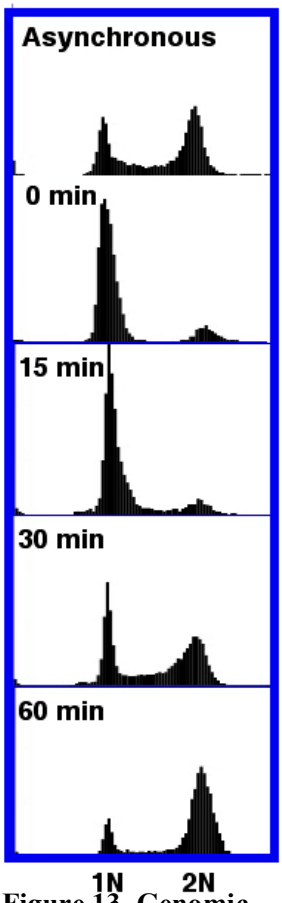

Figure 13. Genomic profiling of synchronized yeast will be utilized to compare the genomes of wild type and mutants strains as they complete their replication cycle. Yeast cultures were synchronized with mating factor and then released. At the indicated time points, samples were fixed, stained with SYBR Green, and analyzed by flow cytometry for DNA content through S-phase.

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