# ENZYMOLOGY OF DNA IN REPLICATION IN PROKARYOTES

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## I. INTRODUCTION

The elucidation of the structure of DNA[1] produced an intuitive argument regarding the mechanism of its replication. Is it known, after 30 years of intensive research, how this is accomplished biochemically? The answer is equivocal. Yes, because classes of enzymes have been discovered that are needed to replicate most DNAs, and it can be demonstrated that the reactions they catalyze must occur in a particular order. No, because it is still not clear how all of these very complex reactions are assembled, coordinated, and controlled, and because additional research keeps producing new and exciting discoveries — protein priming of DNA replication[2] being the most recent of these.

Intensive biochemical studies of DNA replication were initiated in the late 1960s and early 1970s by the nearly simultaneous isolation of *E. coli* strains deficient in DNA polymerase I,[3] of other mutant strains defective in the process of DNA replication (the *dna* genes),[4-6] and the development of crude systems capable of elongating DNA chains in vitro already initiated in vivo.[7-9] This was very quickly followed by the development of more refined crude systems that were capable of faithfully replicating exogenously supplied small DNAs.[10,11] Since that time progress has been explosive, the basic classes of the DNA replication proteins have been established, and the reactions that they catalyze have been detailed. These studies have revealed an enormously complex series of protein-protein, protein-DNA, DNA-DNA, protein-RNA, and RNA-DNA interactions designed to ensure preservation of the genetic material.

A number of excellent review articles have appeared that are quite pertinent. To name just a few, there are those dealing with DNA replication, by Wickner,[12] Ogawa and Okazaki,[13] and Nossal,[14] and of course the omnibus *DNA Replication*[15] and its *Supplement;*[16] the initiation of DNA replication, by Tomizawa and Selzer,[17] and Kolter and Helinski;[18] origin structure, by Hobom;[19] binding proteins, by Coleman and Oakley;[20] helicases, by Geider and Hoffmann-Berling;[21] and topoisomerases, by Gellert,[22] and Liu.[23] In addition there are several on the control of plasmid replication which will have appeared by the time this review is in print.[24-26]

Previous data will not be treated in a comprehensive fashion in this review. However, the system utilized will be described in enough detail to allow one to be able to judge current data in the light of previous studies. Any review article such as this will, of course, reflect the bias and the interests of the reviewer. Thus, the emphasis here will be on the replication proteins and their interaction with the DNA template.

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### A. Basic Principles

One reason for the enzymatic complexity of DNA replication is the inability of DNA polymerase (but not RNA polymerase) to initiate a nascent chain *de novo*. A 3′-OH primer terminus must be provided. This can be accomplished during leading-strand synthesis (the strand made in the overall direction of polymerization) by a nicking event. Extension from a previously existing 3′-OH terminus in the parental duplex leads to strand displacement synthesis if the DNA template is linear, or to its analog, the rolling circle, if a circular DNA is the template. Alternatively, initiation of the leading-strand results from *de novo* synthesis of a primer either by RNA polymerase or by one of the specialized priming enzymes. Initiation of lagging-strand synthesis (the strand made opposite to the overall direction of polymerization) must be *de novo* and must be repeated on the order of once every 1000-5000 nucleotides polymerized. The matter is somewhat more complicated when duplex circular DNA is replicated bidirectionally. The leading-strand template on one side of the origin becomes the lagging-strand template on the other side, and vice versa. Once replication initiates, it is still far from clear, except in a few very specialized cases, how it terminates.

The origin of DNA replication in a particular molecule is defined as the position of the first nascent deoxyribonucleotide residue. This very precise definition must be modified by the observation that there are generally certain DNA sequences required in order to sustain a particular type of initiation of DNA replication, these are called minimal origin DNA sequences. However, in some cases the first residue in the nascent chain may not even be within this sequence, although it is usually very close. It is likely that minimal origin sequences define sites at which some replication proteins must assemble in order to effect initiation. Thus, they gain entry to the template at this point, but may respond to other, more subtle initiation signals somewhere else in the DNA. The most extreme example of this is the primosome assembly site of ΦX174 DNA.[27] This sequence is required for the initiation of ΦX174 single-stranded circular [SS(c)] → double-stranded replicative form (RF) DNA synthesis, but because of the nature of the mobile priming apparatus, initiation can take place anywhere on the chromosome.[28,29] Can this site be called an origin of DNA replication? I propose that, in keeping with the nomenclature of Nomura and Ray,[30] but with greater specificity, the term "primosome assembly site" (pas) be used, followed by one or two letters to indicate its location, to designate such DNA regions. Thus, the site of ΦX174 viral DNA[31] could be pas-X and the site on the H strand of pBR322,[32] pas-BH (see Section IV.A.4).

A considerable number of different types of replication proteins have evolved to help the DNA polymerases in their task. These are

1. The single-stranded DNA binding proteins (helix-destabilizing proteins), which have many functions during DNA replication, including presentation of the template to the polymerase in an acceptable form, and which are also involved in productive interactions with many of the other replication proteins
2. The polymerase accessory proteins, which serve to convert poorly processive polymerases to very highly processive forms, function in increasing the fidelity of DNA replication and may contribute to unwinding of the parental duplex
3. Prepriming proteins and primases, which are responsible for priming DNA replication
4. Helicases, which in an energy-dependent fashion act to unwind the strands of the parental duplex
5. Topoisomerases, which in the case of a duplex circular DNA, are required to relieve accumulated strain, and, in all cases, are required to maintain parental and daughter DNA molecules in their proper topological relationship.

Recent developments in the field have revealed more about how these different types of enzymes perform their function and interact with each other and with the DNA.

## II. BACTERIOPHAGE T7

The complete DNA sequence of T7 DNA, a linear duplex of 40,000 base pairs (bp) with terminally redundant ends of 160 BP, has been determined.[33,34] T7 DNA replication initiates in vivo as an eye form from a point 14.75 to 15% from the left end of the molecule.[35] If this origin is deleted, initiation can occur, although with less efficiency, from a secondary origin located at 4% from the left end.[35] Concatemers are the major DNA forms present late in infection and are processed by a headfull mechanism to yield mature phage DNA.[36,37]

Six-phage encoded proteins, the products of genes 1 to 6, have been shown to be involved in DNA replication by genetic analysis.[38] These proteins are, respectively, an RNA polymerase, a protein responsible for the inhibition of the host RNA polymerase that, in some way, prevents formation of the concatemeric T7 daughter molecules required for phage maturation, an endonuclease, a combination helicase and primase, a subunit of the DNA polymerase that retains the 3′ → 5′ exonuclease, but not the polymerizing activity, and a 5′ → 3′ exonuclease (an independent nuclease not associated with the DNA polymerase).[34,38] Complete activity of the polymerase requires the formation of a 1:1 complex with the host thioredoxin.[39-41] The requirement for other T7 proteins, such as the ligase and single-stranded DNA binding protein, only become apparent if the corresponding host function is inactivated by mutation.[38] In addition, the product of gene 1.2, located between 15 and 16% on the T7 map, may play some role in helix unwinding.[42,43] The phage-specified RNA polymerase is required for initiation at the primary origin,[44,45] whereas the nucleases are required for maturation of the concatemeric daughter molecules.[46] The exonuclease has also recently been implicated in excision of the RNA primers from Okazaki fragments in vitro (see below).[47,48]

The gene 4 protein can, while migrating in a 5′ → 3′ direction on the template strand, unwind short stretches of duplex DNA in a reaction requiring the hydrolysis of (d)NTPs.[49-51] In the presence of ATP and CTP, it will respond to the sequence 3′-CTGGG-5′ or 3′-CTGGT-5′ in single-stranded form on the template strand, by synthesizing the tetraribooligonucleotides pppACCC or pppACCA.[51-56] These primers can be utilized by the T7 DNA polymerase and are preferentially stabilized by the presence of the gene 4 protein.[53] Thus, a combination of T7 DNA polymerase and the gene 4 protein can convert SS(c) ΦX174 DNA to an RF form.[52] Since the direction of movement required for unwinding and repetitive primer synthesis on the lagging-strand template is the same, the gene 4 protein provides two essential functions at the fork. Indeed, a combination of the gene 4 protein and the T7 DNA polymerase can carry out many of the reactions known to be required for T7 DNA replication.

### A. Elongation

Initial studies indicated that T7 DNA polymerase was capable of limited strand displacement synthesis on randomly nicked duplex DNA molecules.[39,40] This synthesis could be stimulated almost 70-fold by the addition of the gene 4 protein in a reaction that required (d)NTP hydrolysis.[49,57-59] The proposal was made that the gene 4 protein was a helicase and was providing for extensive T7 DNA polymerase-catalyzed DNA synthesis by unwinding the duplex ahead of the fork.[59] That this was the case has recently been demonstrated.[50]

Two observations have led to a reexamination of strand displacement synthesis catalyzed by the T7 DNA polymerase and the gene 4 protein. Fischer and Hinkle[44] showed that even in the presence of the gene 4 protein, T7 DNA polymerase purified by their method was inactive on duplex T7 DNA containing nicks. DNA synthesis could be stimulated by adding the T7 RNA polymerase and rNTPs. Under these conditions essentially none of the product was covalently attached to the template. They suggested that there were two forms of the polymerase, the one under study in their laboratory being the more "native", and indicated that the critical factor for isolation of the native form was the presence of EDTA in all of the buffers during purification.

Tamanoi et al.[60] reported the identification of an altered form of T7 DNA polymerase that would not displace the 5′ end of a gap in a duplex molecule during the process of gap repair (the nick could be sealed by DNA ligase). Subsequent studies from Richardson's laboratory have confirmed the existence of two forms of T7 DNA polymerase that were characterized chiefly by their ability to catalyze limited strand displacement synthesis from a nick; form I T7 DNA polymerase could and form II T7 DNA polymerase could not.[61] As previously suggested,[44] the isolation of form II was dependent upon the presence of EDTA in the buffers during purification of the enzyme. Form II could be converted to form I by prolonged dialysis against a buffer lacking EDTA. The rate of interconversion could be accelerated by the inclusion of low levels of $FeSO_4$ or $CaCl_2$ in the dialysis buffer. Form I could not be converted to form II. There is, as yet, no clear indication of the underlying difference between the two forms.

Using a very sensitive assay, Engler et al.[61] have shown that form I was capable of limited strand displacement sythesis, whereas form II (which had a specific activity twice that of the form I used in these experiments) was not. Singly nicked DNA was prepared and the 5′ end of the nick was labeled with $^{32}P$ for these assays. Strand displacement was indicated if the $^{32}P$-label was available for cleavage by alkaline phosphatase subsequent to treatment with the polymerase and T7 DNA ligase. Further evidence of the inability of form II to strand displace was the observation that DNA synthesis on an SS(c) template primed by the gene 4 protein stopped after one round when form II was used, but could continue, by a rolling circle mechanism, for several rounds in the presence of form I.[61]

Using the amount of incorporation of 2-aminopurine catalyzed by the two different forms as an indication of fidelity, Engler et al.[61] observed that form II was roughly twofold more accurate than form I. Yet form I was 50% more accurate than the T4 DNA polymerase, even though they had equivalent levels of 3′ → 5′ exonuclease. This suggests that there may not necessarily be a direct correlation between high levels of 3′ → 5′ exonuclease activity and greater fidelity.

Lechner et al.[62] have developed models to explain template strand switching by the polymerase during strand displacement synthesis. They developed a procedure by which one of the rapidly reannealing forms characteristic of template strand switching could be isolated. Specifically nicked pMB9 form II DNA (produced by limited cleavage of form I pMB9 DNA with the *EcoR1* restriction enzyme in the presence of ethidium bromide) was used as a substrate for form I T7 DNA polymerase. Cleavage of the DNA product synthesized on this template with the *EcoR1* restriction enzyme followed by denaturation released rapidly reannealing double-stranded forms that could be fractionated by gel electrophoresis. These forms could be converted by S1 nuclease treatment and subsequent denaturation into two single-stranded DNAs of approximately equal length. Based upon the DNA sequence of one of these rapidly reannealing forms, the authors suggested that the switch of the template strand was dependent upon the presence of short palindromic segments. The authors proposed two equivalent models, one based on relocation of the primer strand, and one based on strand exchange of the templates, to account for the process. It seems likely that either of these mechanisms can be used to explain the formation of products by strand displacement synthesis of most DNA polymerases. The authors noted that in a similar reaction, only the form I polymerase could resolve (i.e., linearize) Hershey circles formed by annealing T7 DNA molecules digested with exonuclease III so that the terminally redundant sequences were both single-stranded. This raises the possibility that, even though the form II polymerase seems best suited as the replicative polymerase (see below), form I may still be required late in infection to resolve the multimeric T7 DNA concatemers. The mechanism for this switch in activities, if any, remains to be elucidated.

Lechner and Richardson[63] constructed a topologically stable replication fork (Figure 1) and used it to study the differential effects of the gene 4 protein on strand displacement

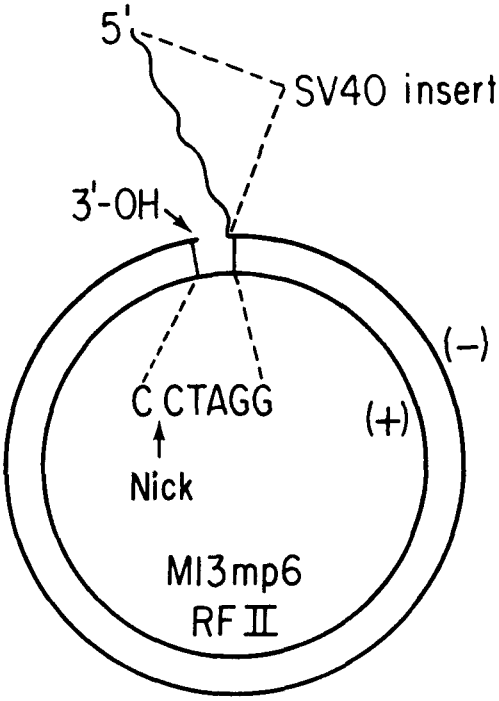


FIGURE 1. Tailed RFII DNA molecule (not to scale). Constructed as described in the text. The size of the nick is exaggerated for clarity. The DNA sequence shown is that of the (+) strand across the site of the nick (i.e., the 3′ terminal nucleotide of the (−) strand is a dGMP).

synthesis by the two forms of T7 DNA polymerase. This substrate was a fully duplex circle bearing a single-stranded 5′ branch that was constructed by annealing the viral SS(c) DNA from phage M13mp6 with the (−) strand from a *BamH1*-digested recombinant M13mp6 RF DNA containing a 237-nucleotide long inserted segment of SV40 DNA. The DNA thus constructed consisted of a fully duplex M13mp6 circle with the SV40 sequences present as a 5′ tail. Since the SV40 sequences were not homologous to the M13mp6 sequences, branch migration could not occur. A limited amount of DNA synthesis (five nucleotides) from the 3′ terminus of the (−) strand would regenerate the *BamH1* recognition sequence present in the phage DNA. Thus, sensitivity of the duplex DNA to the *BamH1* restriction enzyme provided a very sensitive assay for DNA synthesis. This template will be referred to as a tailed RFII DNA molecule in this and subsequent sections.

Only form I polymerase was capable of restoring sensitivity to the restriction enzyme. When considered in the light of the observation that only form I was capable of being stimulated by the gene 4 protein on a singly nicked RFII, this led to the conclusion that form I polymerase was capable of displacing enough of the lagging-strand template to allow entry of the gene 4 protein whose helicase activity subsequently enabled extensive DNA synthesis. Thus, the prediction that form II would be stimulated by the gene 4 protein when the tailed RFII DNA was used as a substrate proved correct. In fact, DNA synthesis was threefold higher in the presence of form II polymerase and the gene 4 protein, than if form I polymerase was used. This assay was also used to demonstrate that the Klenow fragment of *E. coli* DNA polymerase I, but not the intact enzyme, or the T4 DNA polymerase, would strand displace. The gene 4 protein would not stimulate synthesis by these three polymerases, suggesting that the interaction between gene 4 protein and the T7 DNA polymerase was specific. Thus, it is unlikely that in the absence of any added T7 DNA polymerase the gene

4 protein would be capable of completely unwinding the substrate. The gene 4 protein can, most likely, only accomplish extensive unwinding when it is complexed with a T7 DNA polymerase.

Finally, because the size of the strand synthesized using the tailed RFII molecule as substrate was, after cleavage with the *BamH1* enzyme, some multiple of the unit length of the RFII DNA, minimum estimates for the rate of fork movement could easily be calculated. A value of 300 nucleotides per second was determined for the combination of the form II T7 DNA polymerase and the gene 4 protein.[63]

Engler and Richardson[48] have reconstituted T7 lagging-strand DNA synthesis using a combination of a ΦX174 SS(c) template, the gene 4 protein, form II T7 DNA polymerase, T7 DNA ligase, and the T7 gene 6 exonuclease. Both *E. coli* DNA polymerase I and the gene 6 exonuclease were capable of excising primer RNA in hybrid form from the 5′ end of the nascent chains. Using the aforementioned combination of enzymes, conversion of ΦX174 SS(c) DNA to RFI DNA was only 30%, suggesting that a tight regulation of the overall process was lacking. This will, perhaps, be provided when the system can be coupled to leading-strand DNA synthesis. A second activity, in addition to the form II T7 DNA polymerase that allowed conversion to RFI′ DNA when form I T7 DNA polymerase was used, was *E. coli* exonuclease VII. Presumably, this nuclease allowed gap-sealing by degrading any displaced strand before template strand switching could occur.

The helicase and (d)NTPase activities of the gene 4 protein have now been firmly detailed. Matson and Richardson[64] showed that as the length of the single-stranded DNA used to elicit the (d)NTPase activity of the gene 4 protein increased, the $k_{eff}$ decreased; however, the lowest $k_{eff}$ was for an SS(c) molecule. This type of argument has been used previously to imply that the T4 gene 41/61 protein used GTP hydrolysis to move along the DNA.[65] Support for this argument came from the observation that if a small complementary oligonucleotide was annealed to the SS(c) DNA (it cannot be displaced by the helicase activity of the gene 4 protein), the $k_{eff}$ approached that of a linear molecule of equal length. Presumably, this was due to an inability of the gene 4 protein to move continuously around the circular DNA, constantly hydrolyzing dTTP. Since it can only move in one direction,[50] when the oligonucleotide blocks its path it must dissociate from the DNA and rebind in order to continue dTTP hydrolysis.

Further support for the (d)NTP driven translocation of the gene 4 protein along a single strand comes from observations on the frequency of utilization of primer start sites when primer formation was measured directly with ΦX174 SS(c) DNA as a template. Previous studies[51] had indicated that there was a bias in the utilization of primer start sites in the overall 5′ → 3′ direction, suggesting that the gene 4 protein moved in that direction. Matson and Richardson[64] demonstrated that this bias was lost when a nonhydrolyzable (β - γ) ATP analog was used in the reaction. This was explained by the fact that only translocation was inhibited by the analog; but not primer synthesis. Thus, the frequency of utilization of all the sites would be equal under these conditions.

The authors have demonstrated that the rate of dNTP hydrolysis by the gene 4 protein was not affected by concomitant primer synthesis.[64] Since dNTP hydrolysis was directly related to translocation of the protein on the DNA, this implied that the gene 4 protein did not remain bound to the 3′ terminus of the primer after synthesis under the condition described. It had previously been shown that the T7 DNA polymerase preferentially utilized primers synthesized by the gene 4 protein.[52] This suggested that the gene 4 protein may be bound to the primer template during the initial cycles of deoxynucleotide polymerization. Taken together, these two observations are additional evidence for very specific interactions between these two proteins.

Using an assay similar to the one developed by Venkatesan et al.[66] [ in which the displacement of a short (50 to 200 nucleotide) 5′$^{32}$P-labeled DNA fragment annealed to a

SS(c) DNA molecule was measured by polyacrylamide gel electrophoresis] to study the unwinding activity of the T4 gene 41/61 protein, Matson et al.[50] have defined the parameters of the helicase activity of the gene 4 protein. The striking requirement for this activity, that differentiates it from all other helicase activities studied to date, is for a 3′ single-stranded tail on the strand to be displaced from the heteroduplex substrate. This 3′-tail must be a minimum of seven nucleotides long in order to observe unwinding. Since the gene 4 protein moves 5′ → 3′, the authors suggested that the requirement for a single-stranded region may reflect the amount of DNA that is actually single stranded on the leading-strand side of the fork and that the gene 4 protein may bind to both sides of the fork. The tail must be a minimum of 17 nucleotides long if it is on the 5′ end of the fragment to be displaced. Presumably, this length requirement reflects the size of the primary DNA binding site of the enzyme.

This very useful and sensitive assay has now been used successfully to define unwinding in two different systems (T7 and T4). Clearly it should be applied to the *E. coli* DNA polymerizing system and used to examine the helicase activity of the components of the primosome. It also would be interesting to examine the effect on the gene 4 helicase activity of locking T7 DNA polymerase onto a 3′-terminus, which could be accomplished by allowing partial elongation, of another fragment annealed behind the 5′-tailed fragment to be displaced. Would a specific interaction between the polymerase and the gene 4 protein arrest unwinding in the absence of elongation?

### B. Primary Initiation

There are two T7 RNA polymerase promoters, Φ1.1A and Φ1.1B, both oriented for transcription to proceed rightward in the primary initiation region of T7 DNA. Approximately 20 nucleotides downstream from Φ1.1B, and on the opposite strand, so that DNA synthesis would proceed leftward, is a gene 4 protein recognition sequence (i.e., 3′-CTGGT-5′).[67] Fuller et al.[68] have constructed a 75-bp deletion which inactivated both promoters in this region. Electron microscopic examination of these replicating molecules revealed that the primary origin had been completely inactivated. Insertion mutants of Φ1.1A and Φ1.1B, both individually and together, have been constructed and their replication phenotype has been examined.[69] Coinactivation of both promoters resulted in initiation from the secondary origin only. Inactivation of either Φ1.1A or Φ1.1B resulted in an equal distribution of initiation from both the primary and secondary origins.

The question remained, since it was clear that at least one of the promoters was required for primary initiation, if RNA transcribed from these promoters allowed initiation of DNA replication by transcriptional activation, or if the transcripts served as the leading-strand primer. Studies by Fuller et al.[68] showed that the latter possibility appears to be the case. It had previously been demonstrated that linearized plasmids containing the T7 primary origin of DNA replication could be replicated by a combination of the T7 RNA and DNA polymerases and the gene 4 protein.[45] These plasmids were used preferentially over the vector DNA, or other T7 recombinant plasmids containing different promoters than Φ1.1A or Φ1.1B. Replication in this system was unidirectional and to the right when compared to the T7 map.

In order to isolate nascent DNA chains that could be easily analyzed, Fuller et al.[68] have devised a "run-off" assay using as a template plasmid DNA cleaved so that the primary origin region was approximately 600 nucleotides upstream from the right-hand end of the DNA. The size of the major products formed corresponded to the distance from the promoters Φ1.1A and Φ1.1B in the primary origin region to the end of the DNA fragment. The size of these nascent DNA chains decreased by an average of 15 to 20 nucleotides after treatment with alkali, yielding populations with heterogeneous 5′ ends. This indicated, as suggested by Fisher and Hinkle,[44] that RNA transcripts from Φ1.1A and Φ1.1B were utilized as

primers for the initiation of leading-strand DNA synthesis during T7 DNA replication. As expected, no DNA product corresponding to the distance between the gene 4 protein recognition site in the primary origin and the left end of the DNA fragment was observed.

Fuller et al.[68] suggested the following to explain their observations. Rightward leading-strand synthesis is initiated by form II T7 DNA polymerase from a transcript formed by T7 RNA polymerase initiating at either the Φ1.1A or Φ1.1B promoter. It is still an open question if there is any specific processing or specific termination of these primer transcripts. Leading-strand synthesis proceeds rightward and activates the gene 4 protein recognition site by converting it into a single-stranded form. The gene 4 protein binds, synthesizes a primer which is used for leftward DNA synthesis by the T7 DNA polymerase, and moves to the right, coupling leading- and lagging-strand synthesis. The lack of bidirectional synthesis in the replication of plasmids containing the primary origin region of T7 DNA can be accounted for by the lack of a displaced lagging-strand template (the DNA synthesized leftward from the gene 4 protein recognition site in the primary origin, after it passes the Φ1.1A and Φ1.1B promoters, is a leading-strand) which would allow entry of another gene 4 protein. This implies that there are other factors necessary in order to obtain bidirectional replication in the T7 system in vitro.

## III. BACTERIOPHAGE T4

T4 is a linear DNA of 165 kbp that is both circularly permuted and terminally redundant.[70] Replicating DNA purified from cells infected at low multiplicity appear to initiate by two distinct modes. Early in infection, a limited number of eye-type structures can be observed in the electron microscope.[71-73] The number and position of these primary origins are still unclear; it is likely, however, that there are no more than four,[74] and there may only be two.[75] Primary initiation of DNA replication is dependent upon the host RNA polymerase.[76] Late in infection, highly branched forms are observed. These are dependent upon the T4 recombination functions and the T4 type II DNA topoisomerase.[76-78] It has been proposed that these structures are a result of recombinational initiation by a strand from one molecule invading another and acting as a primer.[78]

As expected for a DNA as large as T4, it encodes most, if not all, of the proteins required for its replication. By genetic analysis, 11 gene products have been identified to be involved in T4 DNA replication.[79] These proteins are the products of genes 32, 39, 41, 43, 44, 45, 52, 60, 61, 62, and *dda*. All of the proteins encoded by these genes have been purified, primarily in the laboratories of Nossal and Alberts. Many of the activities required for replication of T4 DNA can be accomplished by different combinations of the phage-encoded replication proteins. Two of the reactions not yet reproduced in vitro are primary and recombinational initiation, although inroads are being made in these directions (see below). The T4 DNA replications proteins have been extensively characterized. T4 DNA polymerase, the 110-kDa product of gene 43,[80] is capable of elongating primers on a single-stranded DNA template.[80] In order to observe gene-43-dependent DNA synthesis on a nicked duplex template, the product of gene 32, the 34.5-kDa single-stranded DNA binding protein, must be added at high concentrations.[81] A large portion of the DNA synthesized under these conditions is rapidly renaturable and presumably reflects extensive branch migration and template strand switching during the reaction. The known properties of the gene 32 protein suggested that it stimulated gene 43 protein catalyzed DNA synthesis by melting the secondary structure of the template strand, thus presenting it to the polymerase in a more suitable form. This was subsequently shown to be correct. Using an SS(c) DNA specifically primed with a $^{32}$P-5′-labeled fragment, gene 43 protein pause sites were evident as discrete bands when the products of the reaction were displayed on denaturing gels. High concentrations of gene 32 protein clearly increased the rate of polymerization by the gene 43 protein and eliminated many pause sites.[82-85]

Additional studies revealed that the gene 43 protein could not function very efficiently on long, single-stranded DNA templates.[86,87] Synthesis could be stimulated by the addition of the 27-kDa gene 45 protein, which functions as a dimer, together with the products of genes 44 (34 kDa) and 62 (20 kDa), which copurify as an 180-kDa complex[88] containing four gene 44 monomers and two gene 62 monomers.[89,90] DNA synthesis was dependent upon all three proteins and at low concentrations of dATP could be stimulated by the addition of ATP, in a reaction shown to be dependent upon ATP hydrolysis. The gene 44/62 protein complex is a DNA-dependent ATPase. Hydrolysis is greatly stimulated by the addition of the gene 45 protein.[91-93] These polymerase accessory proteins were also shown to increase the rate of polymerization by the gene 43 protein and to eliminate pausing in an ATP-dependent fashion.[82,83,85] These five proteins, the products of genes 43, 44/62, 45, and 32 provide the basis of all the T4 DNA replication reactions and are referred to as the "core system".[79] There is some evidence indicating that the effect of ATP is long lasting, i.e., constant ATP hydrolysis was not required in order to observe gene 44/62 plus 45 protein-dependent progression of the gene 43 protein through regions of secondary structure.[83,85] This led Alberts et al. to propose that these proteins functioned as a sliding clamp, serving to keep the gene 43 protein bound to the 3′ end of the nascent chain, thus increasing the overall processivity of the reaction.[83,85]

Studies by von Hippel and colleagues[94] on the effect of the accessory proteins on the processivity of the T4 DNA polymerase support Alberts' proposal. Using the core system and a synthetic primer-template, they determined that only the number of 3′-OH termini elongated was dependent upon the ATP concentration, the extent of elongation was the same, whether or not ATP was present.

Roth et al.[95] have also provided support for the idea of the sliding clamp by studying the effect of the various T4 DNA replication proteins on dNTP → dNMP turnover catalyzed by the gene 43 protein when uniquely primed SS(c) DNA was used as a template. The ratio of formation to incorporation of dNMP was significantly increased by the addition of the polymerase accessory proteins. Since it was known that the gene 43 protein tended to pause at regions of secondary structure in the DNA, they proposed that in the absence of the accessory proteins the gene 43 protein dissociated when it encountered a hairpin; however, in their presence, it was held on the primer terminus for longer periods of time and stuttered, repeatedly excising and incorporating the last few bases in the nascent chain. The dNMP formation to dNMP incorporation ratio also varied inversely with the distance between the initial primer terminus and the region of secondary structure, and was lowered by the presence of the gene 32 protein. A concomitant stimulation of the 3′ → 5′ exonuclease activity of the gene 43 protein was subsequently demonstrated.[96] Bedinger and Alberts (see below)[97] have recently provided similar evidence.

The five-protein core system provides the minimum set of T4 replication proteins capable of strand displacement synthesis.[91,98] At high gene 32 protein concentrations, polymerization approaches the rates observed in vivo. However, it was determined that in order to maintain such high rates of synthesis at low gene 32 protein concentrations, a sixth protein, the 58-kDa product of gene 41, had to be added.[83,99] Subsequent analysis of the properties of this enzyme revealed it to be a helicase[66] and a single-stranded DNA-dependent NTPase that preferred GTP.[65] Arguments based on the stimulation of the GTPase activity by DNAs of different sizes led to the proposal that the gene 4 protein utilized GTP hydrolysis for translocation along the DNA.[65]

Venkatesan et al.[66] demonstrated that the gene 41 protein was a GTP-dependent helicase that moved 5′ → 3′ on the template strand. Stimulation of the core protein system on nicked duplex DNA by the gene 41 protein was preceded by a lag which could be eliminated by first preincubating the gene 41 protein at high concentrations in the presence of GTP.[65] This, plus a sigmoidal dependence of the unwinding reaction on the concentration of the gene 41

protein,[66] suggested that the gene 41 protein functioned as an oligomer, probably a dimer. The combination of the core proteins plus the gene 41 protein is called the core-41 system and can account for T4 leading-strand DNA synthesis.[79]

Priming of lagging-strand DNA synthesis in the T4 system is dependent upon a complex of the gene 41 and 61 proteins.[91,100-102] This complex synthesizes pentaribonucleotide primers of the sequence 5′-pppAC$(N)_3$ or 5′-pppGC$(N)_3$,[83,103-105] however, on duplex T4 DNA containing hydroxymethyl cytosine, only pppAC$(N)_3$ was found.[101] When primer synthesis was measured directly it was found that GTPγS would inhibit the reaction to an extent that correlated with the synthesis of only one primer per circular DNA template.[101] Coupled with the role of GTP hydrolysis in the translocation of the gene 41 protein, this was interpreted as indicating that the gene 41 protein provided a mobile replication promoter for the gene 61 to bind, causing the synthesis of a primer.[101] Therefore, the gene 41/61 protein combination is functionally equivalent to the T7 gene 4 protein. The seven-protein core-41-61 T4 DNA replication system is sufficient to account for both leading- and lagging-strand DNA synthesis.[79,106]

### A. Initiation

Three T4 genes (39, 52, and 60), whose mutant phenotypes (named T4 DNA synthesis delay mutants) are characterized by a reduction in the rate of the initial phase of DNA replication,[107,108] encode proteins of molecular weights of 56, 46, and 17 kDa, respectively.[109-112] Together these proteins form a type II topoisomerase, that, like all type II topoisomerases except DNA gyrase, can relax positively and negatively supercoiled DNA in an ATP-dependent fashion but cannot supercoil form I′ (relaxed, covalently closed duplex) DNA.[110,111] The activity of this protein, and the fact that, in vivo, T4 DNA synthesis in gene 39, 52, 60 mutants did eventually ensue, but now suggested to be dependent upon *E. coli* DNA gyrase,[113] led Liu et al. to propose that the T4 type II topoisomerase played some role in the primary initiation of T4 DNA replication.[110] They suggested that, given a specific DNA sequence-dependent interaction with the primary origin, the T4 type II topoisomerase might be able to induce a localized supertwisting in that region to serve as a site for the initiation of DNA replication.

Localized supertwisting in linear DNAs has been suggested by the electron microscopic studies of Moore et al.[114] on the complexes formed between DNA gyrase and its substrate. They showed that even with linear DNA molecules, DNA gyrase isolated a loop of the DNA. Thus, theoretically, DNA gyrase could topologically constrain a region of linear DNA. Using photobinding of $^3$H-trimethylpsoralen as an assay, Sinden and Pettijohn[115] demonstrated that a region of T4 DNA, corresponding to 20 to 100% of the molecule, became transiently supercoiled very quickly after infection. This transient supercoiling preceded the onset of phage DNA replication. Since this transient supercoiling still occurred when a T4 gene 39 mutant was used for the infection and was inhibited by novobiocin, it was suggested that the host DNA gyrase, and not the phage topoisomerase, was responsible.

Mosig and colleagues[116] have also studied the phenotype of the T4 DNA synthesis delay mutants. They confirmed that novobiocin inhibited the production of progeny phage with these mutants; however, they observed that DNA synthesis was only decreased by 50%. An examination of the production of T4 proteins led them to conclude that the DNA synthesis delay phenotype of these mutants was due to changes in the timing and level of expression of various gene products required for packaging, particularly the DNA ligase (gene 30) and a recombination nuclease (gene 46). Furthermore, they suggested that differences in origin usage and type of T4 DNA replication in the delay mutants could be attributed to the requirement for the activation of various promoters at different origins through changes in the torsional state of the DNA. These observations tend to support the hypothesis of Liu et al.[110] calling for a localized supertwisting at the origin. Additionally, Mosig et al.[116] noted

that in delay *alc* double mutants (the *alc* gene product acts to prevent transcription of cytosine containing DNA and is required for unfolding of the host chromosome), no late gene expression occurred at all. They proposed that the *alc* gene product may be the missing factor that enables the T4 topoisomerase to supertwist the phage DNA.

Kreuzer and Alberts[117] have attempted to study the question of T4 topoisomerase involvement in the initiation of T4 DNA replication directly by determining where on T4 DNA the T4 type II topoisomerase and the host DNA gyrase were bound. Oxolinic acid induces DNA gyrase to make a double-stranded break in a DNA molecule to which it is bound.[118,119] It has been demonstrated that all sites at which DNA gyrase cleaves are also binding sites, but not all binding sites are necessarily cleavage sites.[118-120] Kreuzer and Alberts[117] found that the T4 type II topoisomerase could also be induced to break double-stranded DNA by treatment with oxolinic acid, albeit at a 25-fold higher concentration required for the same effect with DNA gyrase. Thus, the cleavage sites on the template could be mapped. It was also determined that the phage-specified topoisomerase could cleave single-stranded DNA.[121] Cleavage of single-stranded (SS DNA) or cytosine containing native T4 DNA (C-T4 DNA) did not require ATP. However, when glycosylated, hydroxymethyl cytosine containing T4 DNA (glu-HMC-T4 DNA) was used as a substrate, the cleavage specificity increased, i.e., less sites were cleaved. In addition, when this substrate was used in the presence of ATP, the extent of cleavage at these sites increased fivefold. ATPγS mimicked the effect of ATP. Because of the differences in migration of restriction fragments of C-T4 DNA and glu-HMC-T4 DNA, it could not be determined if the sites of cleavage on glu-HMC-T4 DNA were a subset of those apparent on C-T4 DNA. However, the differential effect of ATP was striking. Cleavage by DNA gyrase showed higher specificity than that of the phage topoisomerase; however, it was unaffected by the presence of glu-HMC-T4 DNA or ATP. One of the major T4 type II topoisomerase cleavage sites mapped close to the region defined as the major primary origin (near the strongest T4 early promoter) in the host RNA polymerase dependent primary initiation event.[122] However, the authors reported that they had been unsuccessful in reconstituting eye-type initiation on T4 DNA by using a combination of the core 41-61 system, the phage topoisomerase, the host RNA polymerase, and the phage encoded *dda* helicase (see below).[122] Thus, it is conceivable that the T4 type II topoisomerase may play a role in primary initiation of T4 DNA replication, but just how that occurs is still not clear.

The range of DNA sequence of the T4 topoisomerase cleavage sites[117] seems too broad to impart the specificity required for primary initiation. Thus, if Mosig et al.[116] are correct in their proposal for the role of the *alc* gene product, the required specificity may reside in that protein; through some DNA-sequence-specific binding function it may serve as a preferential loading site for the T4 topoisomerase such that the DNA is wrapped around the enzyme in a defined handedness, enabling it to supercoil.

Formosa et al.[123] have identified two T4 proteins that may be involved in recombinational initiation of T4 DNA replication. They found that both the *uvsX* and *uvsY* proteins, from genetic analysis shown to be involved in recombination, bound to an affinity agarose resin containing the T4 gene 32 protein. The *uvsY* gene product is a 40-kDa single-stranded DNA-dependent ATPase which cleaves ATP to give ADP and AMP in a 2:1 ratio. The *uvsX* protein will catalyze the limited formation of D-loops between homologous DNAs. In addition, in the presence of the *uvsY* protein, the gene 32 protein, and ATP, the *uvsX* protein will catalyze the formation of huge networks of branched DNA molecules. These presumably resemble the recombinational initiation intermediates described by Mosig et al.[78] The networks can be resolved by the *dda* helicase.[123] Effort should be directed at reconstituting DNA synthesis and monomer daughter molecule production using these networks as substrate.

**B. Elongation**

Previous studies[124,125] on the fidelity of T4 DNA synthesis suggested that there was an inverse relationship between the level of the 3′ → 5′ exonuclease activity of the gene 43 protein and the misincorporation frequency on homopolymer templates. In addition, as described above, Roth et al.[95] determined that the effect of the accessory proteins on gene 43 polymerization and dNTP → dNMP turnover was essentially the same, i.e., by acting to keep the gene 43 protein on the primer terminus for longer periods they increased the processivity of polymerization and the ratios of formation to incorporation of dNMP. Bedinger and Alberts[97] have measured the effect of the other proteins in the core system on the extent of 3′ → 5′ exonuclease activity catalyzed by the gene 43 protein on a linearized pBR322 DNA substrate. They found that the addition of the accessory proteins would increase the level of exonuclease activity fourfold. The requirement for ATP in this reaction was similar to the requirement for ATP in the accessory-protein-induced passage of the gene 43 protein through regions of secondary structure,[83] suggesting that a similar phenomenon was occurring. The exonuclease activity of the gene 43 protein alone was stimulated at low levels of gene 32 protein, but inhibited at levels 15-fold higher.[97] The 32*I protein (the amino-terminal fragment of the gene 32 protein) would not stimulate gene 43 exonuclease activity under any conditions. However, in the presence of the accessory proteins, high levels of gene 32 protein no longer inhibited the reaction. The T4 proteins had no effect on the 3′ → 5′ exonuclease activity of the T7 DNA polymerase, suggesting that the effects observed with the T4 gene 43 protein were due to specific protein-protein interactions.[97]

The following interpretation of this data was offered:[97] low concentrations of gene 32 protein stimulated gene 43 exonuclease activity because of a direct protein-protein interaction; high levels of gene 32 protein inhibited by destabilizing the 3′ end of the DNA and binding to it, thus making it inaccessible to the polymerase. The presence of the accessory proteins locked the polymerase onto the 3′ end, once again allowing stimulation by direct protein-protein interactions with the gene 32 protein. Thus, one can conclude, if the level of the 3′ → 5′ exonuclease is a direct indicator of fidelity, that the T4 polymerase accessory proteins may contribute to increasing the fidelity of the polymerization reaction by increasing proofreading. Processivity studies by von Hippel and colleagues,[94] using natural templates, have indicated that dissociation of the gene 43 protein from the primer template is sequence-dependent and that strong terminators (i.e., sequences which caused the gene 43 protein to dissociate) have the capacity to form secondary structure.

In a similar study, Topal and Sinha[126] have reached slightly different conclusions. Misincorporation, using alternating homopolymer primer templates, was measured directly by scoring the turnover of the incorrect base. Based on these measurements they concluded that both the gene 32 and 45 proteins inhibited the selection of incorrect precursors (i.e., turnover of the incorrect base decreased). Titration experiments demonstrated that, with poly dA·dT as a template, as the gene 32 protein concentration was increased, dCTP → dCMP turnover, but not dATP → dAMP + $(pdA)_N$ incorporation, decreased; thus, Topal and Sinha[126] concluded that the gene 32 protein effect was due to its DNA binding activity, and not to an interaction with the gene 43 protein leading to an increase in 3′ → 5′ exonuclease activity. An increase in discrimination due to the addition of the gene 45 protein was attributed to its specific interaction with the polymerase. Interestingly, the gene 44/62 protein had no effect on this system.

At a first approximation, these results seem to contradict those of Bedinger and Alberts.[97] However, it should be noted that Topal and Sinha[126] were not measuring 3′ → 5′ exonuclease activity directly; in addition, in their experiments, polymerization was taking place. One could conclude from this that there are significant differences in the activities of the polymerase, and in its interaction with other proteins, when it is moving 5′ → 3′ as opposed to 3′ → 5′. This is, in fact, supported by Bedinger and Alberts,[97] who noted that the rate of

nucleotide turnover by the gene 43 protein in the core system, when measured in the absence of polymerization, was 25-fold lower than the value predicted from the equivalence of the turnover rate with the synthesis rate during polymerization.

Recent genetic and biochemical studies have indicated a role for the T4-encoded *dda* protein in helix unwinding at the fork. This protein was initially shown to be the major DNA-dependent ATPase induced in *E. coli* after infection with bacteriophage T4.[127-129] Krell et al.[130] purified the 56-kDa protein and showed that it was an ATP-dependent helicase that functioned distributively and required a minimum of a 12-nucleotide long single-stranded tail in order to initiate unwinding.

When infected into an *optA* strain of *E. coli*, *dda* mutant strains of T4 are defective in DNA synthesis.[43] Allele-specific restriction by *optA*$^-$ of the growth of gene 43 mutants was also demonstrated. The lesions of gene 43 mutants that were affected in this manner appeared to cluster near the carboxy terminus of the protein. The suggestion was made that normally the gene 43, 32, and *dda* proteins contributed to unwinding at the fork, and that the *optA* gene product could substitute for an inactive *dda* protein.[43]

Alberts' group has also purified the T4 *dda* protein and determined its effect on replication catalyzed by the T4 proteins.[131-133] Their studies on the activities of the purified protein are in agreement with previous reports.[130] The ATPase activity of the enzyme is not variably dependent upon the substrate length;[132] thus, in agreement with the observed stoichiometry of one enzyme molecule/three nucleotides of single-stranded DNA present,[130] it is unlikely that the protein utilizes the energy derived from ATP hydrolysis for translocation along the DNA. Jongeneel et al.[132] demonstrated that the *dda* protein could resolve (i.e., dissociate), because of its unwinding activity, D-loops formed between a DNA fragment and a SS(c) DNA molecule. Resolution of these D-looped molecules with low concentrations of *dda* protein resulted in the reappearance of the DNA fragment in fully duplex form, whereas the fragment was completely unwound at high concentrations of the protein.

Bedinger et al.[131] have developed a synchronized DNA synthesis system similar to the one used in the T7 system to study strand displacement synthesis. The substrate DNA was fd or M13-pBR322 hybrid RF DNAs cleaved specifically at fd or M13 nucleotide 5781 by the fd gene 2 protein. Synchrony was accomplished by preincubating the replication proteins with only three deoxynucleoside triphosphates (minus dCTP). This allowed polymerization of 12 nucleotides and left the system poised for subsequent extensive DNA synthesis. Progress of the fork could easily be followed by analyzing the size of the DNA product on alkaline agarose gels. It was determined that as little as one *E. coli* RNA polymerase bound to one of the strong promoters on the RF DNA would prevent the progress of the fork for any T4 system tested, even core-41-61. Gene 43 protein was shown to stop about 20 nucleotides behind the RNA polymerase. If the RNA polymerase was allowed to move, the fork would move with it, but at the much slower rate characteristic of transcription. The *dda* protein could overcome the inhibition of fork movement by RNA polymerase by displacing this enzyme from the DNA.

With appropriate DNA constructs, Jongeneel et al.[134] have also shown that the *dda* protein will alleviate the block-to-fork movement provided by an RNA polymerase, in a stationary or transcribing mode, oriented in the direction opposite to fork movement. Thus, it seems likely that the *dda* protein, and, presumably the *optA* protein, is required in order for the fork to pass barriers on the DNA.

Using this synchronized elongation system, Jongeneel et al.[133] demonstrated that the *dda* protein required low concentrations of the gene 32 protein to stimulate fork movement; higher concentrations of the binding protein masked this stimulation. However, the addition of even higher concentrations of *dda* protein restored stimulation, indicating that the gene 32 and *dda* proteins probably competed for the same site on the DNA. The highest extent of DNA synthesis required high levels of both the *dda* and gene 32 proteins, suggesting

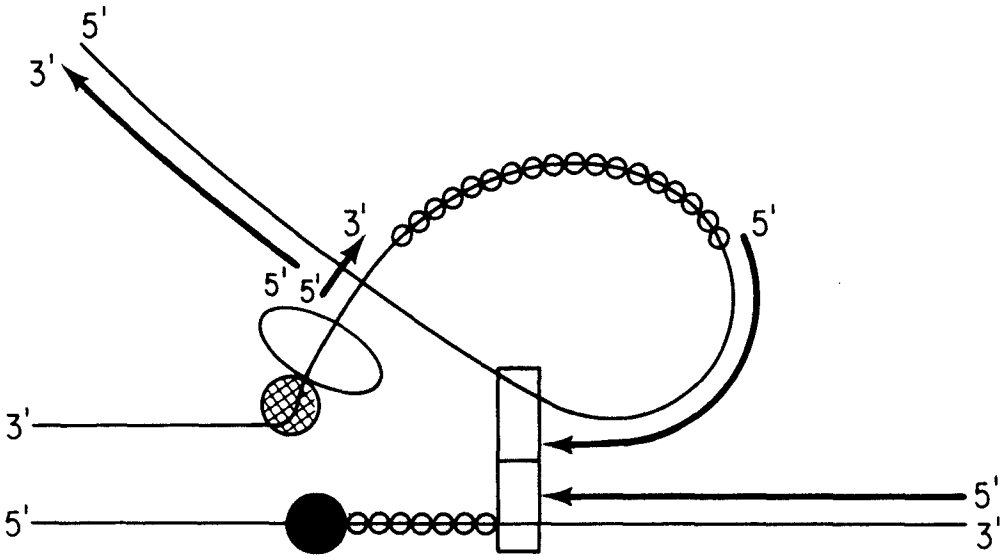


FIGURE 2. One possible distribution of DNA strands and proteins at the fork. The gap between the two Okazaki fragments is exaggerated for clarity. The rectangles represent processive DNA polymerase assemblies, the small open circles single-stranded DNA binding proteins, the open ellipsoid a mobile priming complex migrating 5′ → 3′ on the lagging-strand template, the cross-hatched circle a helicase that can move 5′ → 3′, and the solid circle a helicase that can move 3′ → 5′. Nascent chains are heavy lines and parental chains are light lines. The short nascent chain represents the primer for the next Okazaki fragment that will be synthesized.

that the level of gene 32 protein in some way governed the initiation frequency. This has yet to be tested directly.

There are striking contrasts between the mechanism of action of the *dda* protein and the other T4 helicase, the gene 41 protein.The *dda* protein functions in a distributive manner, is required in stoichiometric amounts for unwinding, and increases the rate of fork movement, but does not eliminate pausing by the gene 43 protein. The gene 41 is catalytic and processive (at the fork), increases the rate of fork movement, and eliminates pausing. Thus, the replication fork becomes even more crowded. In view of their different activities, it is possible that the *dda* protein functions by scaffolding along the phosphate backbone of the DNA, while the gene 41 protein, which may be in intimate contact with the gene 43 protein in order to couple leading- and lagging-strand synthesis (see below), may be oriented toward the helical axis of the DNA molecule.

### *1. Coupling Of Leading- and Lagging-Strand DNA Synthesis*

Now that many replication proteins are available in highly purified form, more subtle aspects of the molecular basis of DNA replication are being addressed. The progress of a replication fork on a replicating DNA molecule is one such problem. Another is whether DNA synthesis on both sides of the fork is coupled. The low abundance of replication proteins, when compared to the number of forks, the need for multiple initiations during lagging-strand synthesis, the dimeric nature of many of the replication proteins, and the very high rates of fork movement in vivo, have led a number of investigators to suggest that synthesis at both sides of the fork is coupled through direct protein-protein interactions.

In the trombone model proposed by Alberts et al.,[122,135] the lagging-strand template DNA is looped out around the polymerase that is polymerizing the nascent lagging strand (Figure 2). This polymerase molecule is coupled by protein-protein interactions to the replication machinery acting on the leading-strand template. The length of the looped-out lagging-strand

template corresponds, when at maximum extension, to the length of two Okazaki fragments. At the time of maximum extension, half of the loop is single stranded and half of it is double stranded. The length of the new Okazaki fragment is governed by the length of the one just previously completed. Synthesis of the new Okazaki fragment presumably stops when the polymerase in some way senses the 5′ terminus of the penultimate fragment.

Alberts et al.[122] have sought evidence that there is coupling of DNA synthesis at both sides of the fork. They reasoned that during short periods of synthesis if there was no coupling, as the gene 43 protein concentration was reduced in the core-41-61 system using randomly nicked T4 DNA as a template, the highly processive gene 43-44/62-45 combination would not dissociate and would produce Okazaki fragments much longer than the average length (1700 nucleotides). In fact, it was observed that the size of Okazaki fragments produced remained the same, supporting the argument that there was coupling of DNA synthesis on both strands of the fork. If all replication forks in the system showed coupling, then the extent of synthesis on each strand should have been equivalent. This was, however, not examined.

Formosa et al.[123] have approached the study of the interactions between the T4 replication proteins in a different fashion. They demonstrated that several T4 replication proteins would bind specifically to an affinity agarose resin containing the gene 32 protein. As expected, the gene 32 protein and the gene 43 protein bound with the highest affinity. T4 proteins that bound with less affinity were the gene 45 protein, gene 46/47 protein (a recombination exonuclease), the *uvsX* and *uvsY* proteins, the *dda* protein, the T4 RNase H, and a 30-kDa T4 DNA binding protein of unknown function. A 32-kDa host protein also bound. This column could be used preparatively to isolate the gene 43 protein. A similar matrix containing the *uvsX* protein bound itself, the gene 32 protein, the gene 42 protein, the *uvsY* protein, and the *dda* helicase.[134] The power of this technique is evident since these very specific interactions were observed when crude extracts from infected cells were passed over the column. Since large amounts of protein are needed to prepare the columns, it may have somewhat limited application in other systems. However, most of the *E. coli* replication proteins, e.g., now can be overproduced in vivo, and large amounts can be purified. Thus, it is likely this technique can even be applied in that system.

## IV. *E. COLI* REPLICATION PROTEINS

### A. Priming of Complementary Strand Synthesis

*1. Priming by RNA Polymerase (fl, fd, M13)*

*E. coli* RNA polymerase can synthesize a short, specific RNA primer on the DNAs of the filamentous phages fl, fd, and M13.[136,137] A region of fd DNA coated with the *E. coli* single-stranded DNA binding protein (SSB), that was protected from nuclease digestion when it was bound to RNA polymerase, corresponded to the region encoding the primer transcript.[138] This oligoribonucleotide could be utilized by the DNA polymerase III system to prime complementary strand synthesis. However, M13 or fl phage engineered with small deletions centered about their unique *Asu I* site could not serve as templates for transcription of this RNA primer, yet the phage were still viable.[139] These microplaque phage yielded tiny plaques and lower titers when compared to the wild type. These data implied that the phage were replicating through secondary initiation sites, or by some nonspecific mechanism. Soeller et al.[140] have demonstrated that the mutant fl microplaque phage replicated in vitro by a ΦX174 primosome type pathway (see Section IV.A.4 below). The level of expression from this origin, that remains to be mapped, is critically dependent upon the level of SSB in the reaction; twice the optimal level (with ΦX174 SS(c) DNA as template) will inhibit it.[413]

There is conflicting evidence addressing the question of whether RNA polymerase and SSB are the only proteins necessary to effect proper initiation from these origins. Crude

extracts that were capable of replicating both ΦX174 and fd DNA would, in the absence of rifampicin, preferentially utilize fd DNA.[10,11] Purified proteins, however, used both templates with equal efficiency.[136,137] The earliest report addressing this discrimination described the purification of a novel RNA polymerase from *E. coli*, RNA polymerase III, that was capable of preferentially priming fd DNA.[141] This RNA polymerase could be induced, in the presence of rifampicin, to release a factor in the size range of 50 to 60 kDa, that would confer differential priming ability upon normal RNA polymerase.

Vicuña et al.[137] reported that the requirements for differential priming included RNA polymerase, SSB, RNase H, and discriminatory factors α and β, with molecular weights of 54 and 18.5 kDa, respectively. Perhaps there was a correspondance between discriminatory factor α and the factor that could be dissociated from RNA polymerase III as reported by Wickner and Kornberg.[141] Vicuña et al.[142] demonstrated that, in the presence of RNA polymerase and SSB, primers were made on both templates that could be degraded by RNase H. The discriminatory factors preferentially stabilized the fd *ori* RNA from this attack.

Recently, Kaguni and Kornberg[143] have indicated that the only factor necessary for discrimination was holoenzyme RNA polymerase — discrimination could be reconstituted with purified core polymerase and sigma subunit. They suggested that the variable results of earlier reports were due to differences in the RNA polymerase preparations. It is difficult to reconcile this explanation with the results described above. The relative recoveries of replication (i.e., discriminatory priming) activity and transcriptional activity in the RNA polymerase purified by Kaguni and Kornberg[143] could not be compared since the latter was not reported for the crude extract.

*2. Priming by the dnaG Protein (Primase)*

The *E. coli dnaG* protein was originally purified based upon its requirement in the rifampicin-resistant conversion of ΦX174 SS(c) DNA to RFII and as a required factor in λ DNA replication in vitro.[144,145] However, the priming function was discovered because of its requirement in the much simpler phage G4 system.[146,147] Similar requirements are exhibited by the DNAs of the α-3, ST-1, and ΦK phages. It was shown that the *dna*G protein was a primase.[147] Subsequent studies have indicated that the enzyme can utilize either ribonucleotides or deoxyribonucleotides as precursors,[148,149] and that the size of the oligonucleotide produced varied dependent upon the mixture of precursors; the higher the concentration of dNTPs, the smaller the primer made.[150,151] ADP could substitute for ATP in the reaction,[149,150] and there was some evidence that it could be incorporated internally.[151]

The precise phage G4 DNA *ori* region was determined by sequencing the single primer that was made by primase in the presence of rNTPs using G4 DNA coated with SSB as the template.[152] The sequence corresponded to a potential hairpin structure within the intergenic space, a region previously suggested to be the (−) strand origin.[146] Based on the level of incorporation of α-$^{32}$P-GMP into primer RNA on α-3 DNA, Benz et al.[151] concluded that primase acted in a stoichiometric 1:1 relationship to the DNA in this system. This reported stoichiometry is interesting in light of the fact that Sims and Benz[153] reported that binding of primase to ΦK DNA at its origin of (−) strand synthesis required the juxtaposition of two regions of the DNA that had the potential to form hairpins. One region was the hairpin complementary to the *ori* RNA, and the other hairpin was 35 nucleotides downstream. Since one primase molecule was not large enough to cover this entire region, the authors proposed that the DNA assumed a specific tertiary structure which was recognized by the protein.

Recently, however, Stayton and Kornberg[154,155] have provided evidence that the stoichiometry of primase to G4 DNA circles is two and not one. The initial velocity of the reconstituted DNA replication reaction (i.e., primase, SSB, and the DNA polymerase III holoenzyme) was shown to be sigmoidally dependent upon primase concentration; significant rates of incorporation were not reached until the ratio of two primase molecules/input circle

was surpassed. Saturation binding of G4 DNA by $^3$H-primase corresponded to a level of two primase/DNA circle and occurred at an input ratio of 10 to 15 primase molecules per DNA molecule. Both SSB and $Mg^{2+}$ were required to suppress nonspecific binding of primase to any DNA. Isolated primase-G4 DNA complexes were fully active for subsequent DNA synthesis. Clearly, in view of this stoichiometry, the data of Sims and Benz[153] will not support the requirement for tertiary interactions in origin structure. There are, however, new studies indicating that primosome assembly sites do require tertiary interactions for activity (see Section IV.A.4).

Stayton and Kornberg[154] have also investigated the fate of primase after primer synthesis. Several lines of evidence indicated that it remained bound to the primer:

(1) Primase was incubated with G4 DNA in the presence of only ATP, UTP, and $\alpha$-$^{32}$P-GTP as precursors. Under these conditions, an octanucleotide primer was formed. When this mixture was filtered through a Biogel column, primase activity could be recovered in the void volume along with the primer-template; the addition of GTP, UTP, and CTP was sufficient to enable extension of the octanucleotide primer to full length (26 nucleotides).
(2) $^3$H-primase bound to primed G4 DNA and isolated by gel filtration was not exchangable with unlabeled primase; however, the primase in this complex would equilibrate efficiently with unprimed DNA when so challenged.
(3) The addition of antibody to primase inhibited the complete SS(c) → RF reaction, even if primer synthesis was accomplished in a preincubation before the addition of the DNA polymerase III holoenzyme.

These data suggest that primase remains bound to the primer after primer synthesis, but that this does not interfere with elongation. Thus, it is likely that there are specific interactions between the DNA polymerase III holoenzyme and primase.

*3. General Priming*

Two systems for priming, analagous to the mobile priming promotors, the T7 gene 4 protein and the T4 gene 41/61 protein, exist in *E. coli*. The simplest may not be of any physiological consequence. Here the *E. coli dnaB* protein, a 50-kDa[156,157] NTPase[158-160] that functions as a hexamer,[156,157] combines with primase to prime any single-stranded DNA template.[161] This system, which is distributive,[162] is inhibited by coating the single-stranded DNA template with SSB.[161,162] Primers made with this system, using ΦX174 as a template, are on the order of 10 to 60 nucleotides in length.[161,162] The addition of the *dnaC* protein stimulates primer production.[414]

An investigation of the mechanism of general priming suggested that bound *dnaB* protein, in the presence of ATP, induced a conformational change in the DNA that stimulated priming by the primase.[163] Binding of primase to ΦX174 DNA could be demonstrated in the presence of the *dnaB* protein, CTP, UTP, GTP, and ATP or App(NH)p; however, the *dnaB* protein was not shown to be required for this binding.[163] Recent studies using tryptic hydrolysis of the *dnaB* protein support the concept of a conformational change induced by the binding of ATP (see Section IV.A.4).

*4. The Primosome and Primosome Assembly Sites*

Early studies on the replication of ΦX174 SS(c) DNA indicated that it proceeded in a rifampicin resistant fashion.[10,11] Subsequent studies, coupled with the study of the elongation of primed single-stranded DNA templates, resulted in the resolution of two of the major replicative assemblies in *E. coli:* the DNA polymerase III multisubunit holoenzyme (see Section IV.B) and the priming proteins that assemble to form the primosome. Studies from

the laboratories of Kornberg and Hurwitz revealed the requirement for eight proteins to effect specific priming of ΦX174 SS(c) DNA.

These proteins are: SSB, the *dnaC* protein (29 kDa),[164] the *dnaB* protein (hexamer of 50-kDa subunit),[156,157] the *dnaG* protein (primase, 64 kDa,[151,165] probably functions as a dimer[154,155]) and four genetically undefined proteins which have been termed proteins i, n, n′ and n″ by Kornberg's group,[166] or replication factors X, Y, and Z (a mixture of two proteins) by Hurwitz's group.[167] In early publications proteins i (for NEM insensitive), and n (for NEM sensitive),[168] or factors X and Y[169] were required for ΦX174 SS(c) → RF DNA synthesis in the presence of the other purified proteins. Subsequently, protein n was resolved into three proteins,[166,170] n (12 kDa, probably functions as a dimer),[171] n′ (78 kDa),[172] and n″ (17 kDa).[173] Protein i (22 kDa)[174] was tightly associated with SSB during purification and probably functions as a trimer.[414] The initial preparation of replication factor Y was clearly resolved into two components, termed factors Y and Z[167] and there was some indication that factor Z could be further resolved into two components termed factor Z and W.[175] The earliest demonstration of any independent activity for these proteins was the elucidation of the single-stranded DNA-dependent ATP (or dATP)ase activity of replication factor Y (factor Y was at this point free of factor Z and W activity).[175] ΦX174 DNA functioned three to six times better as an effector for this ATPase activity than fd DNA. Based on their reported activities and chromatographic data[415] it is clear that protein i = factor X, protein n′ = factor Y, and that a combination of factor Z and W was equivalent to a combination of proteins n and n″. It is not possible to assign the equivalence of these latter two factors more precisely. These names will be used interchangeably throughout the remainder of this section. In addition, the *dnaB* and *dnaC* proteins, and proteins i, n, n′, and n″ are referred to collectively as the "prepriming proteins".

Kinetic data on the synthesis of ΦX174 RFII DNA with purified proteins showed a distinct lag before DNA synthesis commenced. This lag could be eliminated by preincubating the reaction mixture in the absence of dNTPs. It was quickly demonstrated that this preincubation time corresponded to the assembly of a prepriming intermediate on the DNA. Formation of this intermediate required ATP, SSB, and all the prepriming proteins, but not primase. Subsequent elongation, after primer formation dependent upon the *dnaG* protein, did not require any further addition of any of the prepriming proteins. The action of the *dnaG* protein, however, was absolutely dependent upon formation of the prepriming complex.[176,177] This priming complex (i.e., prepriming complex plus primase) has been termed the primosome.[27]

There is substantial agreement on the steps required for assembly of the primosome. Using order of addition experiments followed by the isolation of the intermediates, Wickner[178] proposed that the first event was the binding of factors Y and Z (containing factor W activity) to SSB-coated ΦX174 DNA. This is supported by the observations that: (1) protein n could associate with SSB and form a complex that could be isolated by glycerol gradient sedimentation,[171] (2) at high levels, protein n could bind to SSB-coated ΦX174 DNA;[171] however, it was not clear if this was due to an actual DNA binding event on the part of protein n, or to its ability to associate within SSB, (3) retention of protein n on SSB-coated ΦX174 DNA was significantly stimulated by the addition of protein n′, approaching a level of 1:1:1, protein n′:protein n:ΦX174 DNA.[171] Since, as indicated by its ATPase activity, protein n′ alone can recognize ΦX174 DNA, it is likely that protein n must play a crucial role in the incorporation of protein n′ into the primosome; (4) subsequent addition of proteins i, n″, and the *dnaB*, *dnaC*, and *dnaG* proteins, to the complex described in (3) supported primosome assembly.[171]

Wickner and Hurwitz[167] demonstrated that the *dnaB* and *dnaC* proteins could, in the presence of ATP, form a complex in solution. In this complex, the *dnaB* protein masked the NEM sensitivity of the *dnaC* protein, and the *dnaC* protein suppressed the DNA-

independent ATPase activity of the *dnaB* protein. Kabori and Kornberg[179] and Lanka and Schuster[180] have extended these observations and demonstrated that the stoichiometry of this complex was $(dnaB)_6$ $(dnaC)_6$, monomer:monomer. Complex formation did not occur in the presence of ATPγS. Genetic support for this complex exists since Mauer et al.[181] have isolated *dnaC* suppressor mutations that map in the *dnaB* gene.

It has been proposed that the next step in primosome assembly was, in a reaction dependent upon factor X (protein i), the transfer of the *dnaB* protein from the *dnaB·dnaC* complex to the DNA.[176,182] Under conditions in which there was no coupled elongation, the *dnaC* protein was clearly not present in this new complex, called the preprimosome, that was subsequently recognized by primase to form the primosome, which was capable of primer synthesis. There is data suggesting that in a system in which primosome formation, priming, and elongation are coupled, the *dnaC* protein may remain associated with the primosome.[183] The exact role of protein n″ is not yet clear. The composition of the preprimosome varied dependent upon the method of isolation; different isolation procedures required the addition of different proteins to the system in order to sustain maximum replication rates.[182] Thus, the exact composition and stoichiometry of the proteins in the primosome is ill-defined. Recent reports indicate some difficulty in repeating earlier experiments on primosome formation, and suggest that another factor may be required to maintain the integrity of the primosome.[184]

Complex interactions among the prepriming proteins are supported by the recent demonstration that the *dnaB* protein is composed of two major domains.[185,186] Treatment of the *dnaB* protein with trypsin at 0°C in the presence of ATP resulted in the progressive generation of three major fragments. The first, fragment 1, lacked 20 amino acids from the amino terminus. This fragment was subsequently cleaved to yield fragment 2, a 33-kDa fragment derived from the COOH-terminus (amino acid residues 172 to 470), and fragment 3, derived from the middle of the molecule (amino acid residues 21 to 192). This tryptic fragment data, and the predicted secondary structure of the protein, suggested that fragments 2 and 3 formed two domains connected by a hinge. Fragment 2 fully retained the NTPase, DNA binding, ATP binding, and oligomerization activities of the protein. Only fragment 1 retained any replicative functions, suggesting that fragment 3, along with the 20 amino terminal amino acids, was responsible for interacting with the other replication proteins. ATPγS reduced the rate of cleavage of fragment 1 to fragment 2 and 3, supporting the proposal that ATP binding induces a conformational change in the protein.[163]

Early studies of the primers formed by the primosome indicated that they were complementary to every region of the ΦX174 genome.[28,187] In fact, in the absence of coupled elongation, the primosome can coat the DNA with primers. This feat can, of course, be accomplished in two fashions. The primosome could repeatedly assemble at a site, form a primer, dissociate, and reassemble at a new site or, the primosome could, once on the DNA, move around it, occasionally synthesizing primers. In the latter case, assembly would be site specific. This view was favored in light of the observation that the ATPase activity of protein n′ could be elicited by a specific 55-nucleotide long single-stranded DNA region isolated from the F-G intergenic space of ΦX174 DNA.[31] In addition, only single-stranded DNA fragments containing the protein n′ recognition sequence would catalyze primosome-dependent DNA synthesis.[27]

In experiments in which the gradient of $^{32}$P-label distributed on ΦX174 SS(c) DNA resulting from primer formation by primosomes synchronized at the ΦX174 protein n′ recognition site by incubation in the presence of App(NH)p, was measured, Arai et al.[27] concluded that the primosome migrated in a 5′ → 3′ manner along the template strand. Thus, like the other mobile priming complexes — the T7 gene 4 protein and the T4 gene 41/61 proteins — the primosome would be present on the lagging-strand side of the fork.

Arai et al.[188] compared the effects of ATP, ATP analogs, and dATP on the incorporation of dNTPs into primers by the general priming system and the primosome (uncoupled from elongation by the DNA polymerase III holoenzyme). They found that:

1. Primer synthesis in both systems was promoted by ATP but not dATP.
2. ATPγS promoted extensive primer synthesis by the general priming system but very limited primer synthesis (equivalent to one or two primers per DNA circle) by the primosome.
3. Priming by the primosome in the presence of ATPγS could be stimulated tenfold by the addition of dATP, leading to the conclusion that ATP or dATP hydrolysis was required for extensive priming by the primosome.

Since primosome assembly could occur in the presence of nonhydrolyzable ATP analogs, and protein n′, but not the *dnaB* protein, could hydrolyze dATP, Arai et al.[188] concluded that ATP hydrolysis by protein n′ provided the energy for primosome movement, while the *dnaB* protein was responsible for engineering a DNA region in which primase could function.

Since it could be demonstrated that protein n′ could displace SSB bound to the template DNA,[189] it was suggested that the primosome removes SSB from the region on which a primer will be synthesized and then uses some version of the general priming system during primer synthesis. The actual coupling of the protein n′ ATPase activity to its translocation along the DNA remains to be demonstrated. Once bound, the primosome could survive the successive stages of ΦX174 replication and a synthetic ΦX174 RFI′ containing the primosome could function in ΦX174 RF → RF replication without prior supertwisting.[183]

A recent study[29] supports the idea that while the primers synthesized by the primosome in the absence of elongation were long, in the fully coupled system the primers found on newly synthesized DNA chains were short.[28] RNA primers isolated, by the methods developed by Okazaki's group, from nascent ΦX174 (−) strand DNA produced by purified proteins, were predominantly 4 to 7 nucleotides long. Dinucleotides were also found and these were either pppApdG or pppApG. The 5′-dinucleotides of the longer primers were pppApA, pppApG, or pppApPy in a ratio of 55:39:11. The RNA primers arose from all regions of the ΦX174 genome. This verification of the previous suggestion that the DNA polymerase III holoenzyme could use primers as small as a dinucleotide[28] indicates that there is either some direct interaction between the priming and elongation complexes leading to a stabilization of such small primers, or that once bound to a longer primer, DNA polymerase III will remove some of the RNA in a 3′ → 5′ fashion before deoxynucleotide polymerization occurs. Since DNA polymerase III has no RNase H-type activity, the former possibility is more likely.

Since it is formed solely from host proteins, some of which are clearly required for *E. coli* to replicate its DNA, the primosome is the accepted model of priming of host lagging-strand DNA synthesis. It has, however, only been in the past few years that other systems have been identified that operate using the primosome mechanism. Zipursky and Marians[32] identified two regions of pBR322 and ColEl DNA, that, in pBR322, were on opposite strands and close to the plasmid origin of DNA replication, and were capable of acting as effectors for the single-stranded DNA-dependent ATPase activity of replication factor Y. Nomura and Ray[30] identified a sequence on the L strand of ColEl DNA that was termed rifampicin resistant initiator (*rriA*) that, when inserted into M13 DNA, allowed conversion of the viral SS(c) DNA to RF in a rifampicin-resistant manner. This sequence corresponded to the factor Y effector site found on the L strand of pBR322 DNA. Nomura et al.[190] subsequently confirmed the existence of a second site, *rriB* on the H strand of ColEl.

It was demonstrated that if small restriction fragments containing the factor Y effector sites from pBR322 DNA were cloned into a fl phage vector, they conferred upon that phage

DNA the ability to be converted to an RF form in a rifampicin-resistant, *dnaB*-, *dnaC*-, and *dnaG*-dependent manner.[191] Marians et al.[192] mapped the sites from pBR322 DNA to a 72-nucleotide exonuclease-VII-resistant fragment from the H strand of pBR322 DNA (nucleotides 2114 to 2185) and a 68-nucleotide sequence (nucleotides 2416 to 2350) from the L strand that was not resistant to exonuclease VII. A similar mapping of the ColEl sites is still lacking, although Boldicke et al.[193] localized regions on the isolated strands of ColEl DNA that were capable of catalyzing rifampicin-resistant initiation and presumably corresponded to *rriA* and *rriB*, suggesting that *rriA* and *rriB* were in the same relative position as the pBR322 L and H strand factor Y effector sites.

Small deletions, constructed within the H and L strand factor Y effector sites from pBR322 DNA cloned into fl phage vectors, coinactivated the ability of these sequences to act as effectors for the factor Y ATPase activity, or as rifampicin-resistant, *dnaB*-,*dnaC*-, and *dnaG*-dependent origins of DNA replication.[194] Nomura et al.[195] showed that *rriA* and *rriB*, when cloned into M13 phage vectors, supported protein n′ ATPase activity and DNA replication dependent upon the primosomal proteins. This has also been demonstrated for the pBR322 factor Y effector sequences.[140,196] Thus, it is clear that these regions function as primosome assembly sites. In keeping with the proposal made in the Introduction, the sites present on the H and L strands of pBR322 DNA, the H and L strands of ColEl DNA, and the viral strand of ΦX174 DNA, will be abbreviated as pas-BH, pas-BL, pas-CH, pas-CL, and pas-X, respectively. The site recently detected on the F factor[197] will be abbreviated as pas-F. This term is more specific than the term "rifampicin-resistant initiator", which could also apply to an origin that is dependent solely upon primase (e.g., phage G4).

There is some disagreement whether there is any significant homology between pas sequences. Consensus DNA sequences have been proposed.[197,198] However, it is not clear whether sequence comparisons between pas-BL, pas-CL, and the corresponding L strand sites from a number of ColEl-related plasmids, are fruitful. Because of their role in replication (pas-BL is required in an in vitro system; see Section X.A), position on the DNAs, and overall relatedness of the plasmids, it is unlikely that there would be significant differences between the sites. The recent demonstration that pas-F is homologous to pas-BL and pas-CL[197] could simply be used to argue that the F factor was the progenitor of these plasmids. It is more likely that the important regions of these sites will be identified by mutational analysis.

When pas-BH, pas-BL, and pas-X are compared, the differences are striking. Pas-BH and pas-BL are G + C rich, whereas pas-X is A + T rich.[192] There is little sequence homology between them other than the previously identified hexanucleotide 5′-AAGCGG-3′.[192] In addition, pas-X and pas-BH were resistant to digestion by *E. coli* exonuclease VII, while pas-BL was not.[192] There were also significant differences in the response to variations in the concentration of monovalent ion and divalent cation when recombinant fl SS(c) DNAs containing these sites were used as effectors for the ATPase activity of factor Y.[199]

Greenbaum and Marians[200] have investigated the binding of factor Y to pas-BH, pas-BL, and pas-X using DNase footprinting and methylation enhancement/protection as probes. These studies have indicated that when factor Y was bound to the DNA it protected the entire known length of these sites from nuclease attack. Factor Y mediated protection from methylation of nucleotide residues in these sites occurred in very specific locations that could be mapped to the tops and bases of hairpin structures that could be drawn for these regions. The known stoichiometry of factor Y during the formation of the primosome,[182] and its size (Stokes radius = 36 Å), led the authors to propose that the DNAs were compacted by specific tertiary interactions when they interacted with this protein. This proposal is supported by the isolation of distinct classes of mutations in pas-BH.[196] Over 30 independent single-base changes have been isolated in pas-BH using a new rapid mutagenesis procedure.[201] These mutant sites could be divided into four classes when they were characterized

for their activity as factor Y ATPase effectors and as templates for DNA synthesis using purified proteins.[196] Class I mutants showed no effect in any reaction, i.e., they were silent; class 2 mutants exhibited an altered requirement for $Mg^{2+}$ in the ATPase assay; they were essentially inactive at 1 to 2 m*M* $MgCl_2$ (a concentration at which the wild-type site was fully active) but they could be fully activated by raising the $Mg^{2+}$ concentration to 5 m*M*. This class of mutants displayed slight reductions in replication template efficiency (a maximum of twofold less than the wild type). Class 3 mutations were inactive as factor Y ATPase effectors at any $Mg^{2+}$ concentration and had severely reduced levels of replication template activity (6- to 7-fold less than the wild type). Class 4 mutations behaved in a manner similar to class 2 mutations in the ATPase assay, but they showed significantly reduced levels of replication template activity (3- to 4-fold less than the wild type). Mutations in pas-BL which fall into the first two classes have also been isolated.[140,199]

The characteristics of the class II mutations have been investigated in detail.[199] It was determined that at suboptimal levels of $Mg^{2+}$, the factor Y ATPase effector activity of all three wild-type pas sequences could be stimulated by the addition of monovalent ion. At optimal levels of $Mg^{2+}$, the response to salt varied from site to site. This was also true for the class II mutant sites; however, the levels of $Mg^{2+}$ and salt required to obtain maximal ATPase effector activity were displaced to higher concentrations. These data suggested that the addition of reagents that stabilized duplex structure could alter the conformation of the class II mutant sites so that they were utilized as wild type by factor Y. This suggests that base pairing plays a role in the formation of an active primosome assembly site and supports the idea that they exist in the form of specific three dimensional structures.

The detection of four distinct classes of mutations in a primosome assembly site is not surprising, considering the number of protein-protein and protein-DNA interactions that must take place to effect the assembly of the primosome. Clearly, the existence of this extent of complexity at both the protein and DNA level would enable the control of the initiation of lagging-strand DNA synthesis to occur at a number of different steps along the pathway. These mutant sites should aid in the identification of these control points.

### B. Elongation

DNA polymerase III was identified as the replicative polymerase[202] soon after it was discovered.[203] However, early studies indicated that it was incapable of utilizing long, primed, single-stranded template DNAs.[204] This paradox was solved by isolating different forms of the enzyme that were capable of performing this task. Wickner and Kornberg[205] isolated a large (0.8 to 1 mDa) complex termed the "DNA polymerase III holoenzyme" (herein referred to as "holoenzyme") which could, in an ATP-dependent fashion, copy long single-stranded DNA templates. The holoenzyme could be divided into two components, termed "DNA polymerase III*" (pol III*) and "copol III*", by passage over phosphocellulose.[206] Hurwitz et al.[207,208] reported the isolation of elongation factors I and II that enabled DNA polymerase III, purified by complementation of a *dnaE* temperature-sensitive extract deficient in the replication of ΦX174 SS(c) DNA, to elongate primed single-stranded DNA templates in an ATP-dependent fashion. Subsequently, it was demonstrated that factor II was a combination of the *dnaZ* gene product and elongation factor III.[209]

Many of the subunits of the holoenzyme have been assigned to a number of *dna* genes. The 140-kDa α-subunit is the *dnaE* gene product,[210] the 38-kDa β-subunit is the *dnaN* gene product[211] and is identical to copol III* and elongation factor I. Recent studies have clarified the genetic assignments of the remainder of the subunits. Initially, the *dnaZ* protein was shown to be the 52-kDa γ-subunit[209,212] and the *dnaX* protein the 32-kDa δ-subunit,[213] assumed to be equivalent to elongation factor III.[209] However, it has now been determined that these proteins are products of the τ-subunit that previously had no known genetic locus.[214,215] Since the ε-subunit has recently been assigned to a gene (see below),[216] only the 9-kDa θ-subunit of the holoenzyme remains genetically undefined.

Both Kodaira et al.[215] and Mullin et al.[214] have demonstrated from a genetic and biochemical analysis, that the *dnaX* protein is actually the τ-subunit, now accorded a molecular weight of 78, rather than the 83 kDa originally reported.[217,218] The *dnaZ* protein (the γ-subunit) presumably comprises one terminal 52-kDa portion of the τ-subunit, whereas the δ-subunit presumably corresponds to the other terminal portion of the τ-subunit.[214,215] Plasmids carrying the *dnaZ-dnaX* region directed the synthesis of the τ- and *dnaZ*-subunits, but not the δ-subunit when they were used to program the proteins synthesized by the maxicell technique.[214,215] Treatment of the τ-subunit with very low levels of *Staphylococcus auereus* V8 protease produced a major cleavage product that comigrated with the γ-subunit. Only very low levels of the δ-subunit could be detected under these conditions. The authors indicated that the purification of the δ-subunit was no longer reproducible and suggested that this may be due to its extreme instability.[215]

The question remains if the τ- and γ-subunits are actually independent protomers. No obvious mechanism for their differential synthesis or processing has been revealed by current studies. However, separate, noncomplementing mutations in *dnaZ* and *dnaX* exist,[209,212,213] the *dnaZ* protein can be purified separately from the other components of the holoenzyme,[209,212] and two partial assemblies of the holoenzyme exist that differ from each other by the presence of the γ- and δ-subunits (see below).[206,218,219] In addition, there are cold sensitive mutations, mapping to the *dnaA* gene (*dnaA* SUZ Cs), that specifically suppress the temperature sensitivity of some *dnaZ* mutations.[220] The effect of *dnaA* (SUZ Cs) mutations on the *dnaX* phenotype has not yet been examined. It is possible that the *dnaZ* and *dnaX* alleles simply define two independent domains in the same protein. The physiological significance of the δ-subunit of the holoenzyme is not clear.

The 25-kDa ε-subunit of the holoenzyme has been shown to be the product of the *dnaQ* gene.[216] The *dnaQ* gene is allelic to *mutD*[216,221-223] a previously identified mutator gene that required the presence of thymidine in the medium for phenotypic expression. The *dnaQ* locus abuts the gene for RNase H; although they are transcribed in opposite directions, their transcriptional and translational signals overlap.[222] Tn5 insertion mutations in the shared control region can effect the level of expression of both proteins simultaneously.

The identification of the *dnaQ* gene product as the ε-subunit was based upon comigration of the *dnaQ* protein produced from a plasmid carrying the gene with the ε-subunit of purified holoenzyme in two-dimensional gels.[216] Echols et al.[221] demonstrated that holoenzyme, reconstituted from purified β subunit and pol III* purified from either *mutD* and *dnaQ* strains, was deficient in its 3′ → 5′ proofreading exonuclease activity. This was based on measurements of the extent of removal of a terminal mismatch on a homopolymer primer template and on measurements of dTTP turnover during DNA synthesis in the phage G4 system using the reconstituted holoenzymes. Turnover efficiency with the *mutD5* holoenzyme decreased fivefold, while that of the *dnaQ*29 holoenzyme decreased twofold. These data are supported by the studies of DiFrancesco et al.[224] They demonstrated that some of the activities associated with DNA polymerase III core enzyme purified from a *mutD* strain were significantly altered in comparison to those of the wild-type protein. Some of the most striking differences were: *mutD* core polymerase had fourfold less single-stranded DNA nuclease activity than the wild-type protein, the dNTP → dNMP turnover activity of the wild-type polymerase was twice that of the *mutD* polymerase, and *mutD* polymerase showed increased thermal lability in both its polymerizing and nuclease activities compared to the wild-type protein. Since there is evidence indicating that the 140-kDa α-subunit of DNA polymerase III retains the 3′ → 5′ exonuclease activity,[225] the data of Echols et al.[221] and DiFrancesco et al.[224] suggests that the ε-subunit must moderate the proofreading exonuclease of DNA polymerase III in some fashion. In *Salmonella*, some *dnaQ* alleles can suppress the temperature sensitivity of some *dnaE* mutations.[181] Thus, the long-sought means of creating an error-prone polymerase may be through an ε-mediated modification of the α-subunit of the holoenzyme.

Clearly, the multiple subunits of the holoenzyme function together at the fork, but the existence of a 1-mDa holoenzyme free of the fork is open to interpretation. Purification of intact holoenzyme is difficult, results in low yields, and requires the presence of dimethyl sulfoxide in the column buffers.[217] The *dnaE*, *dnaN*, and *dnaZ* gene products can be purified separately,[211,212,219,226] and partial assemblies of the holoenzyme, differing in their ability to catalyze extensive processive DNA synthesis,[227,228] can also be isolated using different partial assays. Thus, free holoenzyme may be relatively unstable.

The role of some of the subunits and partial assemblies of the DNA polymerase III elongation system in the formation of an actively polymerizing complex has been investigated. Wickner and Kornberg[229] demonstrated that a complex between pol III* (the most complete subassembly of the holoenzyme which lacks only the β-subunit), independently purified β-subunit, and primed single-stranded DNA templates could be isolated by gel filtration. Formation of this complex was dependent upon β-subunit and ATP. Free β-subunit was not required for subsequent elongation of the primers in the complex by the pol III polymerizing activity. Formation of this complex by the holoenzyme has also been demonstrated and was inhibited by antibody to the β-subunit.[217] Johanson and McHenry[230] have demonstrated that once an initiation complex was formed between primed DNA and the holoenzyme, the β-subunit, though it could be demonstrated to be physically present in the initiation complex, was no longer accessible to antibody directed toward it.

Studies by Wickner[231] supported the idea that the *dnaN* gene product was essential for initiation by the holoenzyme. Using purified DNA polymerase III, elongation factors I and III, and the *dnaZ* protein, she investigated the initiation reaction by using order of addition experiments followed by the isolation of intermediate complexes from free subunits and cofactors. She showed that the *dnaZ* protein, reported to have a native molecular weight of 125 kDa (and thus, presumably, a dimer of the 52-kDa γ-subunit) and elongation factor III, reported to have a native molecular weight of 63 kDa (and thus, presumably a dimer of the 32-kDa δ-subunit) formed a complex that eluted from a gel filtration column with an apparent size of 200 kDa. Formation of this complex was not dependent upon ATP or DNA. Incubation of the *dnaZ* protein and elongation factors I and III, in the presence of oligo (dT) primed poly (dA) and ATP, resulted in the transfer of elongation factor I to the DNA. This complex could be isolated free of the other two proteins and ATP. This elongation factor I-DNA complex in the presence of added DNA polymerase III could be utilized efficiently, in the absence of ATP, to catalyze elongation of the template. Since the size of the products made was not determined, definitive evidence that the reaction being observed was that which is characteristic of the highly processive complete DNA polymerase III elongation system was lacking. Since the *dnaZ* protein and elongation factor III were shown to form a complex in solution it was proposed that they operated as a complex during initiation. Considering their recently demonstrated intimate association,[214,215] and the observation that the τ-subunit is a dimer,[218] this is not an unlikely proposition. In light of recent data that excess β-subunit can convert pol III* to an holoenzyme form (see below),[232] it would be of interest to reinvestigate these experiments using singly primed SS(c) DNA as a template and highly purified holoenzyme subunits.

Three subassemblies of the DNA polymerase III elongation system have been described. They are defined by their subunit composition, the extent of processive DNA synthesis of which they are capable, and their response to spermidine and SSB. Pol III* has been described above; DNA polymerase III′ lacks the β-, γ-, and δ-subunits, but retains the τ-subunit,[218] suggesting that the τ- and γ-subunits may have different functional roles; core DNA polymerase III contains only the α-, ε-, and θ-subunits.[219] Processivity decreases as the subunit composition of the enzyme becomes less complex.[227,228] Core polymerase cannot be stimulated by spermidine and is inhibited by SSB.[227] The isolation of the *dnaE* gene on a plasmid[209] should permit the purification of pure α-subunit free of the ε- and θ-subunits and

enable the determination of the role of these smaller subunits in the polymerization and exonuclease activities. DNA polymerase III′ is stimulated by spermidine and is unaffected by SSB.[228] Pol III* is slightly inhibited by spermidine but is stimulated by SSB to make somewhat larger products.[228] In the absence of SSB or spermidine, the holoenzyme makes products of all sizes; in the presence of either spermidine or SSB, the predominant products are very large.[227]

Initiation complex formation has been the subject of several recent studies. Burgers and Kornberg[233,234] demonstrated that ATP formed a relatively stable complex with purified holoenzyme, or with holoenzyme reconstituted from pol III* and purified β-subunit, but not with pol III* or the β-subunit alone. ATP binding could be measured in a nitrocellulose filter assay and a value of 2 to 3 molecules of ATP bound per molecule of holoenzyme was determined. Formation of this complex was competitively inhibited by ATP γS or App(NH)p, supporting previous observations that the nonhydrolyzable analogs could not support extensive synthesis by the complete DNA polymerase III elongation system.[231] If this ATP-holoenzyme complex was formed with radioactive ATP, subsequent exposure to a primed template resulted in a burst of ATP hydrolysis (within 2 to 3 sec). When singly primed G4 DNA circles were used as the primer-template, and the extent of DNA synthesis was measured in parallel to the burst of ATP hydrolysis, it was concluded that two ATP molecules were hydrolyzed for each circle replicated. The use of dApp(NH)P in the reaction mixture precluded the recycling of the holoenzyme in the brief incubation periods used. In light of the observed stoichiometry, the suggestion was made that the burst of ATP hydrolysis corresponded to the formation of the primer-template-holoenzyme initiation complex. Subsequent ATP hydrolysis was not required for replication. If $^{3}$H-β-subunit was used in these experiments it was found to be present in the initiation complex.

Johanson and McHenry[235] have reported the formation of a holoenzyme-primer-template initiation complex in the presence of ATPγS. This effect is clearly not due to an ATP contaminant in the ATPγS preparation since the ATPγS was purified by affinity chromatography on a SH-column. In addition, pretreatment of the purified ATPγS with a level of hexokinase and glucose that would totally inhibit an ATP-dependent initiation complex formation reaction, did not inhibit the ATPγS-dependent reaction. The complexes formed in the presence of ATPγS and ATP were indistinguishable. Using a very sensitive HPLC assay for hydrolysis of ATPγS, they showed that this moiety was, in fact, cleaved by the holoenzyme when it was present in the initiation complex. The inability in previous studies[231,233,234] to demonstrate that ATPγS could substitute for ATP in the reaction may be explained, as suggested by the authors, if previous preparations of the holoenzyme were primarily composed of the lagging-strand version of the enzyme (see below).

A study by Crute et al.[232] has indicated that the ATP requirement for holoenzyme function can be by-passed in yet another fashion. They reconstituted holoenzyme from pol III* and isolated β-subunit and found that the products formed under normal conditions in the presence of ATP, or in the absence of ATP but in the presence of a 10- to 100-fold excess of β-subunit, on poly (dA)·oligo (dT) were indistinguishable. Stable initiation complex formation required ATP. However, with an excess of the β-subunit full-length material could be formed even in the absence of ATP. This may indicate that the affinity of the polymerase for the 3′ terminus might change depending on the presence of forward (i.e., polymerizing) movement. It would be interesting to determine if a stable complex could be formed in the absence of ATP with excess β-subunit on singly primed SS(c) DNA after partial elongation was allowed in the presence of three dNTPs. The authors suggested that the holoenzyme existed in equilibrium with pol III* and β-subunit. This is supported by the finding that even the most purified preparations of holoenzyme yielded products that were characteristic of pol III*.[227] The addition of ATP shifted the equilibrium toward the holoenzyme. Excess β-subunit was also capable of accomplishing this by mass action. It has also been demonstrated

that excess β-subunit is capable of converting core DNA polymerase III to a more processive form.[416]

In these experiments, and others which deal with the interrelationship between the subunits of the holoenzyme, enzyme concentrations are determined on a functional basis; e.g., the molar amount of holoenzyme in the experiments of Burgers and Kornberg[233,234] was quantitated on the assumption that one holoenzyme molecule converted one G4 DNA circle to an RF form under a defined set of condition. Similarly, in the experiments of Crute et al.,[232] the excess β-subunit added was 10- to 100-fold in excess of the amount of β required to saturate a given amount of pol III* in the G4 SS(c) → RF system. While this type of determination yields an apparent active concentration of the protein in question, it is not clear how the actual stoichiometry of the proteins involved can be determined by measurements such as these.

*1. Coupling of Leading- and Lagging-Strand DNA Synthesis*

Attention has been directed in the *E. coli* system, as it has in the T7 and T4 systems, toward the question of how leading- and lagging-strand DNA synthesis is coupled. In the *E. coli* system this becomes even more of a problem because of the stability of the holoenzyme complex on the DNA. Burgers and Kornberg[236] showed that holoenzyme isolated in an initiation complex with singly primed G4 DNA would, in the presence of dTTP, dCTP, dGTP, and dApp(NH)p, convert the SS(c) DNA to an RF form within 2 min. When challenged, after the first round of synthesis was over, with new, primed G4 SS(c) DNA, no additional incorporation took place unless ATP was present. If additional ATP was provided, the second round of DNA synthesis took twice as long, indicating that the holoenzyme recycling time was about 2 min. Considering the frequency of initiations required on the lagging-strand side of the fork, and the total number of holoenzyme molecules present in the cell, this is far too slow to account for the very high rate of DNA replication in a rapidly growing *E. coli*, assuming a single origin of replication and bidirectional growth.

A number of groups have studied the dissociation of the holoenzyme from primer-templates. In a study of pausing by the DNA polymerase III assemblies, La Duca et al.[237] demonstrated that 65% of the pause sites could be correlated with regions of secondary structure. The strongest pause sites had the sequence 5′-TTTA-3′ adjacent to a region with the potential of assuming secondary structure. Pausing by the holoenzyme was relieved, to a large extent, by SSB, but only if it was added to the template before the holoenzyme. Some pause sites were still observed in the presence of SSB. These corresponded to the strongest pause sites observed in the absence of SSB and may represent natural pause sites or preferred dissociation sites. In addition, paused holoenzyme would not, when challenged, utilize a high-density primer-template. This suggested that the paused enzyme had undergone some change such that its normal activities had been altered. How this relates to holoenzyme molecules about to be recycled on the lagging-strand template remains to be determined.

In a related study, Baumel et al.[238] investigated the effect of a Tn5 insertion on fd SS(c) → RF DNA synthesis. Tn5 contains long terminal direct repeats which will, in single-stranded form, reanneal to form a stem-loop structure that is readily visible in the electron microscope. Complementary strand synthesis initiated from the fd *ori* RNA by the holoenzyme would not advance through the Tn5 insertion. Instead, replication eventually started nonspecifically at a number of sites. The addition of the *rep* protein allowed the holoenzyme to proceed through the Tn5 hairpin, presumably by unwinding it. This unwinding was still not fast enough to enable complete replication of the DNA and prevent secondary initiations. These two studies confirm the high degree of stability of a holoenzyme bound to a primer terminus.

On the other hand, it is clear that the holoenzyme can recycle after it has completed replicating the G4 DNA circle. Burgers and Kronberg[236] have investigated this recycling

and have attempted to find conditions that will influence this event. By isolating a complex of replicated G4 DNA bound to the holoenzyme (i.e., after the first stage in the experiments described in the first paragraph in this section), they determined that the presence of the gap in the DNA itself did not alter the recycling time. Addition of DNA polymerase I and DNA ligase was also without effect. However, the recycling time could be decreased significantly by allowing transfer of the holoenzyme to take place intramolecularly (i.e., between two RNA primers bound to the same G4 DNA). Several different species of primed G4 DNA were prepared that had differing average amounts of RNA primers annealed to them (1 to 9.8 primers per DNA circle). Initiation complexes were formed at limiting concentrations of holoenzyme. Replication of each different species occurred in the same amount of time. Since it was demonstrated that the average size of the DNA product formed on the multiply primed G4 DNA corresponded to the length of the DNA divided by the number of primers annealed to it, it was concluded that the holoenzyme was recycling from primer to primer on the G4 DNA. The calculated recycling time in this system was 2 to 5 sec, significantly faster than the previously demonstrated time of 2 min, but still not fast enough to account for lagging-strand DNA synthesis of the *E. coli* chromosome.

It is possible that the holoenzyme cycling time could be reduced even more in a system in which leading- and lagging-strand synthesis are coupled. Johanson and McHenry[235] have proposed that this coupling may be accomplished through the use of two distinct forms of the holoenzyme, each of which functions exclusively on only one side of the fork. They noted that ATPγS caused the dissociation of one half of the holoenzyme-primer-template initiation complex formed in the presence of ATP. The reverse was also true, i.e., the extent of initiation complex formation in the presence of ATPγS was 50% of the amount of complex that could be formed with the same amount of holoenzyme in the presence of ATP. Thus, they postulated the existence of two forms of holoenzyme, one that could form an initiation complex in the presence of ATPγS, and one that could only utilize ATP for initiation complex formation. Initiation complex formed by the holoenzyme that could utilize only ATP could be dissociated by ATPγS. They suggested that the dimeric holoenzyme molecule acting at the fork to couple the synthesis of both strands, was an asymmetric dimer. The holoenzyme synthesizing the leading strand was the one which could form a complex with ATPγS. The holoenzyme synthesizing the lagging strand, which needs to dissociate repeatedly, was the one that could only form an initiation complex with ATP. This is an intriguing proposal.

There is, in fact, evidence supporting this proposal. Holoenzyme bound to fd DNA specifically primed at two locations, results in the formation of looped DNA structures visualized by electron microscopy. Loops are not observed if core DNA polymerase III, even in large excess, is used.[416] The implication is that one dimeric holoenzyme molecule is binding to the 3′ ends of both primers to create the loop. It is also clear that if ATP binding is required to dissociate the holoenzyme from the primer terminus,[233-235] in order to account for extensive processive synthesis in the presence of ATP (and dATP), one must reason that an holoenzyme that is moving forward (i.e., polymerizing) is insensitive to the effect of ATP and that only paused holoenzymes can dissociate. The observation by Burgers and Kornberg[234] that ATPγS caused dissociation of greater than 90% of the preformed holoenzyme initiation complex, can be explained in this lexicon by their preparations of holoenzyme being primarily the lagging-strand version postulated by Johanson and McHenry.[235]

### C. Helix Unwinding

A class of proteins has been characterized that couples the energy of ATP hydrolysis to the unwinding of the duplex DNA strands. These enzymes differ in the direction in which they unwind DNA, their stoichoimetry in the unwinding reaction, the extent of a single-stranded DNA starter region they require in order to initiate unwinding, the effect of SSB

on the unwinding reaction, and the utilization of nucleoside triphosphates other than ATP. The known helicases are: helicase I,[239] II,[240] and III,[241] the *rep* protein,[242] the T4 *dda* protein,[130] and presumably, the product of the *E. coli optA* gene (see Section III.B). The *rep* gene product will be described in more detail in the next section and the *dda* (gene product) and the proposed activity of the *optA* gene product have already been described.

The mechanisms of these proteins have been discussed recently,[21] so they will not be addressed here. The major progress in this area has been the assignation of helicase I as the *traI* gene product of the *E. coli* sex factor F[243] and helicase II as the *uvrD* gene product.[244-247] The identification of helicase I as a F encoded protein effectively rules out a significant role for it in chromosomal DNA unwinding; however, the identification of a pas sequence on F,[197] and the demonstration that F encodes its own SSB, termed SSF,[248,249] raises interesting possibilities about F replication. Does F encode its own DNA polymerase or other DNA replication proteins?

Antibody to helicase II was shown to inhibit both chromosomal DNA synthesis in a cellophane disc system, and ColEl DNA synthesis in a crude extract, by 50%.[250] Temperature-sensitive mutants that were thought to be in the gene for helicase II,[251] however, had no effect on ColEl DNA synthesis. These mutants have now been definitively mapped to the *uvrD* gene,[252] although they did not show any UV sensitivity or mutator phenotype characteristic of classic *uvrD* alleles. It is likely that these seemingly contradictory results can be explained by the number of interactions that helicase II has with other replication and repair proteins.

Helicase II, which functions 5′ → 3′, and the *rep* protein, which functions 3′ → 5′, probably contribute the bulk of the unwinding activity at the fork, an activity on each side. This is supported by the extent of inhibition of chromosomal DNA synthesis by anti-helicase II (50%),[250] and the twofold reduction in the rate of fork movement in *rep* mutants of *E. coli*.[253] Additionally, *rep uvrD* double mutants apparently cannot be constructed.[254] The appropriately engineered conditional lethal double mutant has not yet been generated. Thus, definitive proof that the *rep* protein and helicase II are both acting at the fork is still lacking.

Taucher-Scholz et al.[247,254] have suggested that the reason that there are two helicases, one on each side of the fork, is primarily to ensure that the fork will move past DNA damage on either side. It is possible that *uvrD* mutations are more UV sensitive than *rep* mutations due to two contributing factors: (1) it may be more difficult for the *rep* protein to advance through the damaged duplex DNA region than it is for helicase II — this is not unreasonable considering the difference in stoichiometry between the *rep* protein and helicase II. Since the *rep* protein is catalytic and highly processive it may be easier to dislodge it from the DNA. Helicase II, on the other hand, functions essentially stoichiometrically and thus may be able to simply push through damaged regions of the duplex. (2) Repair on the lagging-strand template, since it is opposite to the direction of fork movement, may be inherently more difficult than repair on the leading-strand template.

It has also been demonstrated that helicase II is UV-damage inducible.[255] There are two promoters upstream of the *uvrD* gene, one of which contains a *lexA* binding sequence that can account for its transcriptional control.[256] Induction of SOS functions resulted in a threefold increase in the expression of β-galactosidase placed under the control of the *uvrD* promoters. If the rate of duplex DNA unwinding is directly proportional to the level of helicases in the cell, and if the elongation machinery is normally constrained from moving at its highest possible speed by the unwinding rate, this raises the possibility that the increase in spontaneous mutation rate associated with the SOS system may be, in part, attributable to the rate at which the holoenzyme polymerizes DNA.

## V. INITIATION AND TERMINATION OF ΦX174, fl(fd) AND M13 VIRAL STRAND DNA SYNTHESIS

Genetic studies indicated that the ΦX174 gene A protein, and the gene 2 protein from fd and M13 phage, were required for the production of progeny RF and viral strand circular DNA.[257-259] The ΦX174 gene A protein was originally purified using radiochemical identification as an assay and shown to be a site-specific endonuclease that cleaved ΦX174 DNA in the viral strand of the A cistron.[260,261] The subsequent development of crude systems in vitro capable of supporting ΦX174 and fd RF → RF DNA synthesis[262,263] enabled the purification of the ΦX174 gene A[262,264] and the fd gene 2 protein by complementation assays.[265]

The requirements for ΦX174 viral strand production proved to be very different from those for complementary strand synthesis.[242,266] This was revealed by the discovery that a simple system containing ΦX174 RFI DNA, the ΦX174 gene A protein, the *rep* protein, SSB, and the holoenzyme, was capable of producing progeny (+) strand circles in amounts that were far in excess (five- to tenfold) of the input RFI DNA. It was suggested that the nick induced by the ΦX gene A protein served as a primer for a rolling-circle-type mechanism in which new unit length viral strands were generated, clipped, and presumably ligated into a circle, by the ΦX174 gene A protein.[266]

The ΦX174 gene A protein becomes covalently attached to the 5′ end of the nicked ΦX174 RFII DNA (RFII·A complex).[264] This complex could be isolated by sucrose gradient centrifugation after treatment of ΦX174 RFI DNA with the ΦX174 gene A protein. Isolated RFII·A complex was as active a template for viral SS(c) production as intact RFI DNA. At low concentrations of the ΦX174 gene A protein, RFI′ DNA, that could be converted to RFII by treatment with proteinase K, could also be isolated. This suggested that the ΦX174 gene A protein might function in a manner similar to a type I topoisomerase.[264]

The steps in the production of progeny SS(c) could be further divided into two partial reactions. Scott et al.[242] showed that in the presence of the ΦX174 gene A protein, SSB and the *rep* protein ΦX174 RFI DNA was unwound from the point of the gene A nick, yielding an SS(1) molecule attached to the gene A protein, and a (−) strand SS(c) molecule. It was reported that gel filtration purified gene A·SS(1) complex could be sealed to form an SS(c) in the presence of $Mg^{2+}$.[267] It was proposed that the *rep* protein associated with the gene A protein that was bound to the 5′ end of the nick and acted to unwind the DNA, while SSB was required to prevent reannealing of the unwound strands. It was suggested that to account for its apparent breakage and reunion activity, the gene A protein had two active sites. In catalytic viral strand synthesis, there was a concerted cleavage of the newly generated gene A protein recognition sequence resulting in transfer of the gene A protein to a new 5′ terminus, coupled to circularization of the displaced unit length viral strand.

It was also suggested,[267] and later demonstrated in reconstitution experiments,[268] that one could account for duplex ΦX174 RFI DNA replication by a combination of the two known enzymatic pathways, the SS(c) → RF pathway dependent upon the primosomal proteins, and the RF → SS(c) pathway described above. Presumably, as the viral strand is displaced by the *rep* protein unwinding reaction, pas-X becomes active, a primosome assembles and catalyzes initiation of (−) strand synthesis on the displaced (+) strand. Reinberg et al.[269] demonstrated that the same pathway was also followed in crude extracts prepared from *E. coli* mutants thermosensitive in the *dnaB*, *dnaC*, and *dnaG* gene products. Heat-inactivated extracts produced only circular viral strand DNA when supplied with ΦX174 RFI DNA and the gene A protein. However, when the corresponding wild-type *dna* gene product was also supplied, the products were a mixture of RFI and viral strand circles. Recircularization of the displaced (+) strand must occur before synthesis, which may initiate at or near pas-X, reaches the 5′ end of the (+) strand, or before the primosome assembled at pas-X migrates

past the 3′ border of the gene A recognition sequence on the displaced (+) strand. Minus-strand synthesis is inhibited when the ΦX174 capsid proteins accumulate and form a precursor of the phage head. Viral strand synthesis becomes coupled directly to the maturation cycle of the phage heads. The assembly of viral peptides onto the nascent (+) strand SS(c) DNA prevents exposure of pas-X in an active form.[270-272]

The ΦX174 gene A product is a multifunctional DNA replication protein. It is convenient to consider its activities as a number of discrete steps:

(1) Recognition: the initial binding event of the gene A protein to its recognition sequence on ΦX174 RFI DNA.
(2) Cleavage: a gene A protein catalyzes site-specific endonuclealytic cleavage between nucleotides 4304 and 4305 resulting in its the covalent attachment to the 5′ end of the (+) strand (RFII·A complex). Steps 1 and 2 are required for initiation of ΦX174 (+) strand viral DNA synthesis.
(3) Unwinding: the *rep* protein associates with the gene A protein in a manner that allows the *rep* protein to catalyze unwinding of the RFII·A complex from the gene A protein induced nick. Concomitant with this, the holoenzyme catalyzes DNA synthesis using the 3′-OH terminus of the nicked (+) strand as a primer.
(4) Termination: the gene A protein encounters the recognition sequence that has been regenerated by DNA synthesis and once again cleaves it while also resealing the displaced (+) strand to form an SS(c) molecule. In a concerted fashion the gene A protein is transferred to the 5′ end of the nascent (+) strand. After this last event, known as reinitiation, the cycle repeats.

Assays have been developed to study each of these steps individually and they are described below.

The laboratories of Baas and Jansz have investigated the sequences required for initiation by studying the gene A protein catalyzed cleavage of isolated oligonucleotides containing the gene A recognition sequence.[273] The sequence required for cleavage by the gene A protein varied, depending upon how the DNA was presented to the protein. These studies used as a reference point the 30-nucleotide conserved region present at the viral (+) strand origins of the related icosahedral phages ΦX174, G4, U3 and ST-1[274-276] that, when cloned into plasmid vectors, could be cleaved by the ΦX174 gene A protein.[274] The ΦX174 gene A protein cleaves between the seventh and eighth nucleotide residues (the first position corresponds to the 5′ end of the conserved sequence). Oligonucleotides comprising nucleotides 1 to 10 and 1 to 16 could be cut;[273] however, duplex oligonucleotides corresponding to positions 1 to 16 or 1 to 20 could not be cleaved when cloned into a plasmid.[277] Site-specific mutagenesis indicated that some nucleotides within positions 7 to 14 may not be required.[278] Brown et al.[279] have shown that nucleotides 1, 29, and 30 were not required for nicking, although nucleotide 1 was required for efficient template utilization.

The existence of three domains within the conserved sequence has been proposed.[273,275,276] The first domain corresponds to positions 1 to 10 and might be considered the minimal ΦX174 gene A protein recognition sequence. The second domain, based on the finding of silent mutations within this region, is of indeterminate length spanning nucleotides 11 to 16. The third domain, called the "key sequence", corresponds to positions 16 to 30. This domain is required for cleavage in a supercoiled DNA. In their model, the gene A protein interacts with the key sequence first, inducing a localized unwinding (which is aided by the A + T rich nature of the second domain), exposing the first domain in single-stranded form and allowing cleavage.

Reinberg et al.[280] investigated the termination reaction by using plasmid DNA containing two copies of the ΦX174 viral (+) strand origin. When both sites were present in the same

orientation, the RF → SS(c) protein system produced two circular products, one large and one small, corresponding to the sizes predicted by the distances between the two ΦX174 gene A cleavage sites. When the sites were oriented in the opposite sense, no small SS(c) molecule could be detected. More importantly, the same products could be produced from the vector containing the two viral strand origins in the same orientation in the absence of the holoenzyme. These data suggested that both sites were cleaved with equal frequency. Exposure of the second site, either by unwinding or unwinding coupled to DNA synthesis, resulted in termination and circularization.

Brown et al.[281] have extended these studies by limiting the amount of extension that could take place by the holoenzyme. Starting with unlabeled RFII·A complex, extension in the presence of ddGTP and the other three dNMPs (including α-$^{32}$P-dATP) regenerated the gene A protein recognition sequence to position 25 of the conserved sequence. Similarly, if ddCTP was used instead of ddGTP, the recognition sequence was regenerated only up to position 18 of the conserved sequence. Only elongation in the presence of ddGTP enabled reinitiation and termination to occur. Reinitiation was measured by the detection of a $^{32}$P-oligonucleotide linked to the 59-kDa gene A protein moiety on SDS-polyacrylamide gels. Termination was measured by the detection of the original viral strand (of the RFII·A complex) as an SS(1) DNA, indicating that no circularization had taken place and that the viral strand had been displaced by the unwinding reaction, or as an SS(c) DNA, indicating that the displaced viral strand had been circularized. The findings of Reinberg et al.[280] and those described above, indicate that the requirements for termination and reinitiation are the same — the regeneration of at least the first 25 nucleotides of the conserved region. However, the sequence required for initiation of (+) strand viral DNA synthesis probably has to extend past nucleotide 25 since it has been demonstrated that plasmids containing nucleotides 1 to 25[283] or 1 to 26[417] of the conserved sequence are not nicked by the gene A protein. The demonstration by Eisenberg et al.[267] that unit length SS(1)·A complex could be resealed, may be represent an actual reversal of the endonuclease activity.

The oligonucleotide-ΦX174 gene A protein complex described above was analyzed.[282] It was determined that the protein was linked to the predicted DNA sequence and that the linkage was a tyrosine-phosphoester bond. Van Mansfeld et al.[284] have reached an identical conclusion. Roth et al.[282] found that proteolytic digestion of micrococcal nuclease treated $^{32}$P-oligonucleotide-linked gene A protein (isolated as described above) yielded two distinct peptides linked to the mononucleotide. Thus, as originally proposed by Eisenberg et al.,[267] there are two sites on the gene A protein capable of being covalently bound to the 5′ end of the cleavage site. Mechanistically, this suggests that termination and reinitiation requires the presence of two gene A recognition sequences, the one originally cleaved and the newly regenerated one, within the catalytic site of the gene A protein.

Studies by Meyer and Geider[285] revealed that the gene 2 protein of phage fd functioned essentially in the same fashion as the ΦX174 gene A protein. These two proteins have been compared in detail in a recent review.[286] The major differences are that the fd gene 2 protein does not become covalently attached to the nicked viral strand, and will not cleave in a site-specific manner single-stranded DNA containing the gene 2 recognition sequence, although it will cleave such a single-stranded sequence present at a replication fork.

Studies from Zinder's[287] group have indicated a similar arrangement of sequences at the viral (+) strand origin of phage fl as there are at the ΦX174 viral (+) strand origin. These studies have utilized a number of different assays coupled to deletion mutational analysis of the sequences involved. These assays are: (1) nicking of fl sequences inserted into plasmid DNAs by the gene 2 protein as an indication of the recognition sequences; (2) fl directed stimulation of the replication of plasmids carrying fl viral (+) strand origin sequences, as an indicator of a functional fl viral strand origin; (3) synthesis of single-stranded plasmid DNA from species containing one or two copies of the fl viral (+) strand origin sequence,

that is packaged into fl phage particles in cells superinfected with fl helper phage and can be differentiated from wild-type fl because of the antibiotic resistance markers transduced along with them, as an indicator of termination.

These analyses[287-291] have contributed to the following scheme of the fl viral strand origin. The nicking site is located at nucleotide 5781. The DNA sequence required for nicking in vitro extends 4 nucleotides upstream and about 20 nucleotides downstream of the cleavage site. This is called the "core" sequence, or A region. The sequence required for termination coincides with the core sequence on the 3′ end, but extends 12 nucleotides upstream of the cleavage site. The termination signal includes the upstream portion of a palindromic sequence in which the gene 2 recognition site is inbedded. It is thought that since the gene 2 protein does not remain bound to the DNA, this sequence is required for termination in order to bring together, in a hairpin, the two halves of the cleaved site. The signal required for efficient fl directed replication shares the same 5′ boundary as the nicking recognition site, but extends 83 nucleotides downstream of the cleavage site. This region (the B region) can be subdivided into an additional two regions. The B2 region corresponds to the 3′ terminal 40 nucleotides of the B region and must be present in *cis*, although it can apparently be separated from the cleavage site by large insertions, in order for efficient replication to occur. The B1 region corresponds to the 5′ portion of the B region and might simply be a spacer sequence.

Dotto and Zinder[292] investigated the following paradox: fl phage containing large insertions at the B1/B2 junction (the site of most recombinant DNA inserts) functioned normally, yet viral (+) strand origins derived from these phage could not be complemented when they were present on a plasmid by wild-type fl phage. Only the homologous recombinant phage would complement. flR218, a recombinant cloning vector containing a 4-bp insertion at the B1/B2 junction could also complement. In fact, flR218 could complement either the viral origin of any recombinant phage containing an insertion at the B1/B2 junction, or the A domain alone when it was present on a plasmid. Since plasmids containing the A domain could also be complemented by other plasmids that had been engineered to overproduce the gene 2 protein, this suggested, and it was verified, that flR218 carried a regulatory mutation leading to the overproduction of the gene 2 protein. Surprisingly, these mutations mapped to gene 5. A similar analysis has led to the definition of a "replication enhancer site" in the M13 viral strand origin corresponding to the fl B2 region.[293] Based on interference experiments, it was suggested that this region may bind gene 2 protein. Dotto and Zinder[292] have proposed that the B region may be the site where the replication fork forms. Raising the gene 2 concentration may alter some of the protein-protein or protein-DNA interactions required for initiation, eliminating the requirement for the B region.

It is possible that the fl and M13 B2 region may correspond to the key region in the ϕX174 viral (+) strand origin. Applying the model of Heidekamp et al.[275] to the fl system would result in the proposition that there is a high-affinity DNA binding site for the gene 2 protein within the B2 region that cannot be cleaved by the protein. This site would have a higher affinity for gene 2 protein than the actual cleavage site. The B1 region would correspond to the second domain in the ϕX174 viral (+) strand origin. Binding of the gene 2 protein occurs at the B2 region site, enabling cleavage at the recognition sequence (the A region), either by direct transfer of the protein, or by protein-DNA interactions. At higher concentrations, the gene 2 protein can bind directly to the recognition sequence and cleave it.

Data obtained in these systems represents the best studies to date on termination in any DNA replication system. Unfortunately, these are highly specialized cases, although there are probably analogous reactions during strand transfer in conjugal mating. Detailed biochemical studies on *E. coli* chromosomal-type replication termination awaits the reconstitution of theta type replication systems in vitro with purified proteins. At that time, plasmids

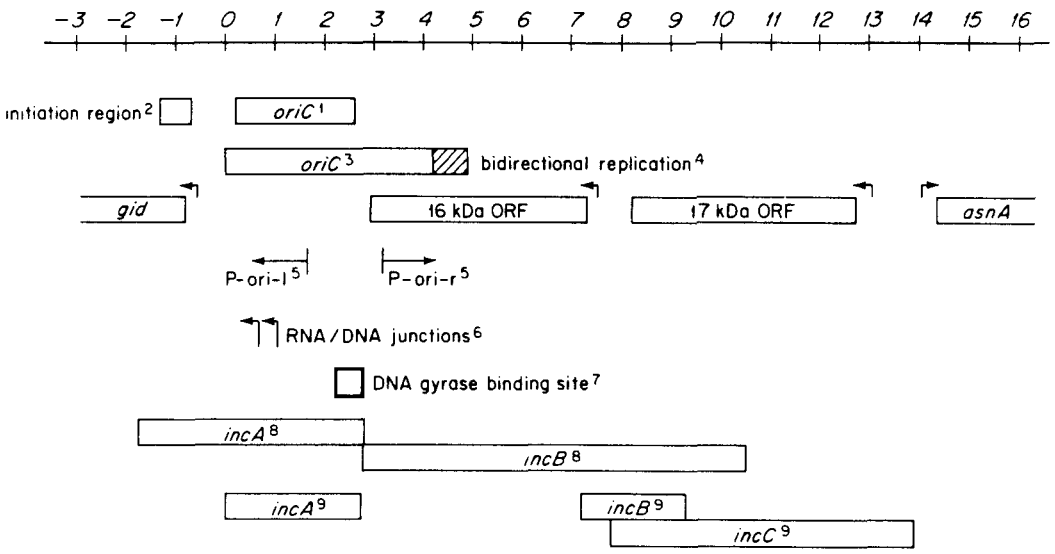


FIGURE 3. The *oriC* region of the *E. coli* chromosome. The scale, in hundreds of base pairs, is according to the standard *oriC* DNA sequences.[298,299,301] Known genes and open reading frames are marked and their promoters and direction of transcription indicated by arrows. The various features are described in the text and are as reported by Oka et al.,[297] Tabata et al.,[331] von Meyenburg et al.,[302] Messer et al.,[303] Lother and Messer,[308] Hirose et al.,[309] Lother et al.,[347] Yamaguchi et al.,[314] and Stuitje and Meijer.[313]

could be constructed and studied that contain the well mapped *E. coli* or *B. subtilis ter* regions.

## VI. *oriC*

There has been considerable progress in the understanding of DNA replication that originates at *oriC* since its isolation by recombinant DNA technology.[294-296] A 245-bp DNA sequence has been defined as the minimal origin sequence[297] (positions 23 to 265 of the standard *oriC* sequence, the top strand, as generally written, is 5′ → 3′ and corresponds to the genetic clockwise sense)[298,299] based on the ability of hybrid pBR322-*oriC* plasmids to transform *polA* strains. A similar analysis of the *Salmonella typhimurium oriC* sequence gave essentially the same results.[300] This methodology has been questioned in the past,[301] and will be discussed below. On the other hand, deletion analysis of minichromosomes formed from the *oriC* region and containing a *bla* gene indicated, with the viability of a given minichromosome as a measure of the dispensable nature of the deleted sequence, that the minimal origin was from nucleotides 1 to 422.[302] Further analysis indicated that sequences to the right of position 422 were required for efficient bidirectional replication in vivo.[303] The difference between this minimal origin sequence and the smaller one is still not clear; however, the 245 bp sequence is usually the one referred to as "minimal". pCM959, an *oriC* containing minichromosome derived from positions −677 to +3335 of the *oriC* region, has been completely sequenced.[304]

DNA sequence analysis and promoter mapping by a number of groups has resulted in the evolution of the following picture of the *oriC* region (Figure 3).[298,299,304-307] The first known genetic marker clockwise from *oriC* is the *asnA* gene. The open reading frame (ORF) corresponding to this protein begins at position 1434. Transcription initiates near nucleotide 1410 (promoter C1[307] and probably PR3[306]) and proceeds rightward. Two ORFs are located between *oriC* and the *asnA* gene. One codes for a 17-kDa protein whose ORF begins at position 1282 and terminates at position 827. Transcription begins near position 1306 (pro-

moter p2[305] and C2[307]) and proceeds leftward. The second codes for a 16-kDa protein whose ORF begins at position 734 and terminates at position 297. Transcription begins near nucleotide 758, proceeds leftward (promoter pl,[305] B,[307] and PL2[304]) and has been shown to terminate efficiently within the 245-bp minimal *oriC* sequence.[305] However, no evidence could be gathered indicating that either the 17- or 16-kDa protein promoter was required for *oriC* function. A third promoter that transcribes leftward has been found just counterclockwise of *oriC* (promoter p3,[305] E,[307] and PL1[306]) and probably accounts for transcription of the *gid* gene, encoding a 70-kDa product whose ORF begins at one of two ATGs at nucleotide −73 or −89, and the ORF encoding a 28-kDa protein to the left of the 3′ end of the 70-kDa ORF. The *atp* operon begins at about −4.2 kbp. It is also transcribed leftward. All these proteins encoded by plasmids carrying the appropriate regions of the *oriC* environs have been detected in maxicells. Morita et al.[306] have identified two other rightward transcribing promoters, PRI, located near position 286, and PR2, located between position 658 and 973.

Using transcription in vitro of restriction fragments containing *oriC*, Lother and Messer[308] have identified two promoters within *oriC* whose transcripts could be used to prime DNA replication. One promoter, P-ori-r, is transcribed rightward, with transcripts initiating predominantly at positions 313 and 323. This promoter probably corresponds to the promoter PR2, identified by Morita et al.[306] A second promoter, P-ori-l, initiates at position 166, is transcribed leftward and the corresponding transcript is contained entirely within the minimal *oriC* sequence. Transcripts from both P-ori-l and P-ori-r terminate after approximately 110 nucleotides.[308a] Thus, the termination points of transcripts from P-ori-l correspond to the RNA/DNA transition points identified by Okazaki's group.[309] Using promoter fusion vectors, Ganea and Zyskind[418] have recently detected a strong transcription terminator on the counterclockwise strand of the *Klebsiella pneumoniae* minimal *oriC* sequence.

It is likely that the different reaction conditions, RNA polymerase preparations, and methods of analysis used in the studies summarized above, are the reason for the apparent disparity in the results. Standardizing the system would be beneficial.

DNA sequence analysis of *oriC* regions cloned from five different enteric bacteria has indicated that there is considerable homology between them, and has enabled the derivation of a consensus bacterial origin sequence.[310] The most striking aspect of this sequence is the conservation of 9 of 11 *dam* methylase sites (5′-GATC-3′) within the 245 bp minimal origin sequence of *E. coli*. This finding has led to speculation about the role of methylation by the *dam* methylase in the initiation of replication at *oriC*. In fact, as determined by Buhk and Messer,[304] the tetranucleotide GATC is repeated 49 times on pCM959 DNA, and even when the *oriC* sequence is deleted, it still is the tetranucleotide that appears most frequently in the DNA sequence of the region.

Mutational analysis has been directed at the 245 bp minimal *oriC* sequence. *oriC*$^+$ function is scored as the ability of pBR322-*oriC* hybrids to transform a *polA*$^-$ strain. Sodium bisulfite induced saturation mutagenesis of the G·C bp within the *oriC* region indicated that 70/101 G·C pairs of the minimal *oriC* sequence could be altered without affecting *oriC* function.[311] Mutations in 17 of the remaining 31 G·C pairs affected *oriC* function to varying degrees. Inactivation of *oriC* function appeared to be additive (i.e., two mutations on the same molecule were functionally more restrictive than one), and those G·C pairs that exhibited this phenotype fell primarily within the consensus minimal *oriC* sequence. The finding that the same regions of *oriC* could tolerate single-base substitutions, but not insertions and deletions, the mapping of these regions to the consensus *oriC* sequence and the reasoning that different replication proteins had to be spaced properly on *oriC* for full activity, has led Asada et al.[312] to propose that there are two regions in *oriC;* recognition sequences for protein binding, and spacer sequences that are required to keep the replication protein binding sites at the proscribed distance.

Recently, analyses have been reported attempting to detail some of the controlling functions (i.e., *inc*, *par*, *ccd*, *sop*) of the *oriC* region. These analyses have been complicated by the lethality of overexpression of the *atp* genes, and the subtle differences in copy number between various *oriC* containing plasmids. There is agreement that the region around *oriC* that is under examination does not contain a *par* sequence similar to the ones that have been identified on F and pSC101.[24] It is reasonable to argue that the *oriC* region should resemble the region around *oriS* on F (see Reference 24), since the behavior of the F factor mimics that of the *E. coli* chromosome. However, the only type of controlling regions that have been identified thus far near *oriC* have been those that contribute to the expression of incompatibility.

Plasmids carrying the region around *oriC* are relatively stable in *recA* strains, although even minichomosomes derived from the original 9 kbp *EcoRI oriC* fragment segregate plasmidless cells at a measurable frequency. *oriC* plasmids can express incompatibility toward other *oriC* containing plasmids, but not to the chromosome itself.[313] Two groups have investigated this phenomenon and agree that one incompatibility region (*incA*) corresponds to *oriC* itself.[313,314] *incA* is located either between positions 1 to 276[313] or −178 to 285.[314] Yamaguchi et al.[314] identified a second region, *incB*, corresponding to positions 280 to 1050. Either *incA* or *incB* expressed incompatibility when present on a high copy number plasmid. Stuitje and Meijer[313] have succeeded in dissecting *incB*, as defined by Yamaguchi et al.,[314] into two overlapping segments, *incB*, corresponding to positions 723 to 934, and *incC* corresponding to positions 777 to 1495. However, unlike Yamaguchi et al.,[314] they found that none of the *inc* regions could act independently. *IncB* and *incC* will function if both are together on the same plasmid, or if either one is present on the same plasmid carrying *incA*. *incA* and *incB* could be separated by 4 kbp and still express incompatibility.[313] The determination of whether *incA* and *incB*, or *incA* and *incC*, had to be present in *cis*, was not reported. The authors have suggested that *incB* corresponds to the promoter for the 16-kDa protein, while *incC* corresponds to the gene for the 17-kDa protein and its promoter.

The minimal *oriC* region (*incA*) contains four potential *dnaA* protein binding sites[315,316] and the p1 promoter sequence contains one (see below).[315] Stuitje and Meijer[313] suggested that *incA* and *incB* expressed their functions by titrating the *dnaA* protein, and they proposed that the 17-kDa protein (*incC*) functioned by enhancing *dnaA* protein binding to a *dnaA* recognition sequence [i.e., *incA* (*oriC*) or *incB*]. As the authors indicated, if this were the case, *incA* and *incB* should have individually expressed incompatibility. Their failure to do so may indicate that they were not present at a high enough copy number. Patterns similar to this have emerged for the replication control of a number of plasmids (see Reference 24), so that it should not be surprising to encounter it in this system as well. However, interpretation of these data is complicated by two additional observations: (1) the promoter of the *dnaA* gene itself also contains a *dnaA* recognition sequence.[315] It has been proposed that the *dnaA* protein exerts a negative autoregulatory function at this locus.[316] Thus, the level of *incA*, *incB*, and *incC* in the cell will effect the level of the *dnaA* protein as well. Churchward et al.[317] have reported that a *lacZ-dnaA* promoter fusion which lacks the *dnaA* recognition sequence and overproduces the *dnaA* protein, has no effect on DNA replication of *dnaA*$^+$ strains. However, the fusion construction did have a sequence just 5′ of the *dnaA* coding sequence which was a seven out of nine nucleotide match with the proposed *dnaA* consensus recognition sequence.[316] (2) Stuitji and Meijer[313] observed that small deletions in the 16-kDa ORF had no effect on minichromosome stability, whereas insertions in the same place did. This may imply a requirement for transcription across the left-hand border of the 16-kDa ORF into *oriC*, as the transcript from p1 is known to do.[305] How this relates to incompatibility function [recall *incA* (*oriC*) and *incB* (p1) could be separated by a large insertion], or to replication control, remains to be determined.

Early genetic analysis indicated that the initiation of replication from *oriC* required the products of the *dnaA*, *dnaI*, *dnaP*, *and dnaB* and *dnaC* genes, as well as RNA polymerase.[318]

The complexity of the interactions between these proteins was first indicated by the isolation of mutations in the β-subunit of RNA polymerase that could suppress the temperature sensitivity of some *dnaA* alleles.[319] The interaction between these proteins, predicted from the genetic data, has been borne out by the biochemical analysis of DNA replication from *oriC* (see below). Other extragenic suppressors of *dnaA* mutations have also been isolated and, for one, the protein involved was identified by recombinant DNA techniques as a 68-kDa species which had to be present in high copy number in order to be effective.[320]

The *dnaA* protein clearly interacts with, or can be affected by, the activity of a number of other proteins in addition to RNA polymerase. Blinkowa and Walker[321] investigated the effect of the *dnaA* (SUZ Cs) mutation on other *dna* genes. They determined that this *dnaA* allele could not be transduced into *dnaC* or *dnaG* temperature-sensitive strains, even at the permissive temperature. It could be transduced, but at very low frequency, into *dnaB* and *dnaE* temperature-sensitive strains. The *dnaE dnaA* (SUZ Cs) combination was demonstrated to be lethal and it was presumed that the other combinations were also lethal. This suggests that under normal circumstances the *dnaB*, *dnaC*, *dnaG*, and *dnaE* gene products interact with the *dnaA* gene product, and that they cannot interact with the *dnaA* (SUZ Cs) gene product. In light of the requirement for these *dna* gene products in elongation, the authors proposed that the *dnaA* protein functioned during elongation in a replication complex containing multiple copies of itself and other replication proteins.

Another protein that appears to interact with the *dnaA* protein is DNA gyrase. Previous studies had indicated that *dnaA*46 mutants were hypersensitive to coumermycin, an inhibitor of DNA gyrase. Filutowicz and Joncyk[322] demonstrated that this hypersensitivity could be suppressed by second-site mutations in *rpoB*. They were also able to demonstrate that, in a *dnaA*$^+$ background, rifampicin-resistant *rpoB* mutations could be isolated that suppressed the temperature sensitivity of two different *gyrB* alleles.[323] It is conceivable this reflects some defect in the template preparation and activation steps proposed by Fuller et al.[324] to be required for replication from *oriC* (see below). Some other observations that may reflect a defect in this step were made by Tanaka et al.[325] and Rasmusaen et al.,[326] who isolated mutations that mapped to *rpoC* that appeared to have uncoupled the initiation of chromosomal replication from the origin/cell mass coupling ratio constraint. The copy number of the *oriC* region of the chromosome was increased by roughly a factor of two in these mutants. The copy number of *oriC* containing minichromosomes increased two- to threefold. These results imply that there is some rifampicin-sensitive step mediated by RNA polymerase that may be rate limiting during the initiation of replication at *oriC*.

Another factor governing initiation frequency, at a step just after the ones described above, could be the availability of the *dnaG* protein. Using λ-transducing phage carrying the *dnaG* gene, Wold and McMacken[326a] demonstrated that (1) maximal expression of primase required sequences far upstream of the gene and (2) transcription through a strong terminator 30 to 70 bps in front of the primase gene was modulated by antitermination. In addition, based on transcriptional and genetic data, they proposed that the *dnaG* gene formed an operon with the neighboring structural gene for the RNA polymerase sigma subunit, *rpoD*. Subsequently, Burton et al.[327] and Lupski et al.[328] have shown that the *rpsU*, *dnaG*, and *rpoD* genes, in that order, form an operon. Three promoters are upstream of these genes, and it has been proposed that a *lexA* protein-binding sequence is also present.[328] The operon contains two terminators, one between *rpsU* and *dnaG* (t1), and one at the end of the operon (t2).[327] It has been proposed that an *E. coli* equivalent of the λN gene product functions in the regulation of gene expression from this operon.[326a,328] The relative copies per cell of each protein indicates that this regulation is very complex. However, it is of great interest that the expression of three key proteins in the three major metabolic processes in the cell appear to be jointly regulated.

The development of an in vitro soluble enzyme system capable of faithfully replicating *oriC* containing minichromosomes has been reported.[329] A dependence upon RNA poly-

merase, DNA gyrase, SSB, the *dnaB* protein, and high concentration of the *dnaA* protein could be demonstrated. This system required the presence of a hydrophilic polymer. Replication was also dependent upon an active *oriC* sequence. Dideoxythymidine triphosphate titration experiments, coupled with electron microscopic observation of the replication intermediates, indicated that replication proceeded bidirectionally from *oriC*,[330] although it could be argued that there was a preference for replication to proceed leftward from *oriC* first, before it started in the rightward direction. The *oriC* sequence on the vector used, M13*oriC*26 RF DNA, was interrupted at position 422. The region downstream from position 422 had been proposed to be required for bidirectional replication.[303] The authors point out that Messer et al.[303] may have been observing a very infrequent event.

Using the same soluble system and similar ddTTP experiments, Tabata et al.[331] have concluded that the 245-bp minimal *oriC* sequence directs the initiation of bidirectional replication just to the left of itself. This effect was dependent upon the use of a *dnaA* complementing fraction in the replication system. The region where initiation occurred was localized to between 67 to 122 nucleotides to the left of *oriC*. These DNA sequences derived from, in three different plasmids, three different regions of pBR322 DNA. Since the data of Kaguni et al.[330] was not mapped as precisely as that of Tabata et al.,[331] these two observations are certainly compatible. However, the findings of Tabata et al.[331] do appear to contradict those of Hirose et al.,[309] who mapped two leftward RNA/DNA transition points to positions 71 and 108 of the *oriC* sequence. They could not detect any rightward RNA/DNA transition points near *oriC*. The data of Hirose et al.[309] are supported by the location of the termination of transcription from P-ori-1, as reported by Lother et al.[308a] Tabata et al.[331] suggest their observations may be accounted for by the presence of an RNA primer that begins within the *oriC* sequence and is not elongated by a DNA polymerase until it has been extended to the left of the *oriC* sequence.

The initiation points that have been detected, at the nucleotide level, by Hirose et al.,[309] are for leading-strand DNA synthesis in the leftward direction. Primosome assembly sites, the obvious candidates for lagging-strand origins, are not immediately apparent, i.e., the 9-kbp *EcoR1* restriction fragment carrying *oriC*[294] will not, when denatured, elicit the site-specific ATPase activity of factor Y.[415] However, Kaguni et al.[332] demonstrated that both strands of a restriction fragment spanning nucleotides 1 to 3500 of the *oriC* region were capable, when the fragment was cloned into an M13 phage DNA vector, of sustaining replication of the chimeric phage DNA in a *rep*3 host, indicating the presence of complementary strand origins on the fragment. It is likely that these are the same signals recently detected by Stuitje et al.[333] They used a ΦX174 gene A protein-dependent transduction system[198] in which packaging into phage of plasmid sequences contiguous with the ΦX174 gene A protein recognition site was dependent upon the presence of a complementary strand origin in the contiguous sequence. Five such regions between coordinates 2100 to 3200 of the *oriC* sequence were detected, three on the counterclockwise strand and two on the clockwise strand. Efficient replication in vivo from these origins was dependent upon the *dnaB* protein. The DNA sequence of these origins shared homology with the hexanucleotide sequence 5′-AAGCGG-3′ previously shown to be common to pas-BH, pas-BL, and pas-X.[192] Although the authors[333] suggested that these origins were primosome assembly sites, such an assignment should be demonstrated directly by established techniques.[195,196] The location of these sites at such a considerable distance from *oriC* suggests a novel mechanism of initiation of synthesis of the lagging-strand near *oriC*.

The *dnaA* protein is a 52-kDa monomer that tends to aggregate.[316,334] Both the aggregated and monomer forms are active, although the specific activity of the monomer is three- to fourfold greater than that of the aggregate. Maximum replication rate was elicited at an input of 15 *dnaA* molecules per DNA template.[316] The *dnaA* protein will bind *oriC* containing DNA specifically.[316,334] The affinity for RFI DNA carrying *oriC* was four- to fivefold greater

than it was for RFIII DNA. With the use of ColEl DNA as a competitor, conditions could be found where M13*oriC*26 RFI DNA, but not M13*oriC*26Δ221 RFI DNA (containing a deletion of *oriC* sequences 21-352), was bound by the *dnaA* protein. Binding of the *dnaA* protein to *oriC* protects the *Hind* III site within the minimal sequence from digestion by that restriction endonuclease.[316]

It is likely that the *dnaA* protein binds to a DNA sequence, identified by Hansen et al.,[315] that is repeated four times within the minimal *oriC* sequence, once at the promoter for the 16-kDa protein just to the right of *oriC*, and once in between the two promoters for the *dnaA* gene. Fuller et al.[316] have proposed a consensus *dnaA* protein binding sequence, 5′-TTATC(A)CACA-3′. They indicated that it was also present at the origin of replication of the plasmid pSC101, that had previously been reported to be dependent upon *dnaA* for its replication,[335] the $IR_L$ sequence of Tn5 and between the origin of pBR322 DNA replication and pas-BL on the L strand. They proposed that the *dnaA* protein acted positively in certain cases — at *oriC* and *ori*-pSC101 — and negatively in other cases — at *ori*-pBR322, *ori*-ColEl and the *dnaA* gene promoter.

Another unusual DNA sequence present at *oriC* that has received considerable attention is the repeated GATC sequences. It is interesting to note that the *dnaA* gene promoter region also has a high number of GATC tetranucleotides (9 in 225 bp).[315] Recent evidence suggests that it is unlikely that methylation by the *dam* methylase at these sites plays a direct role in *oriC* dependent replication. Marinus et al.[336] isolated Tn9 mediated insertion mutants of the *dam* gene that have no detectable level of *dam* methylase activity in crude extracts. These strains are quite viable; however, they do show an increase in spontaneous mutation frequency, indicating that previous proposals that *dam* methylation directed mismatch repair may be correct.[337] Forteire et al.[338] examined the replication of *oriC* containing plasmids purified from *dam*$^-$ strains in crude extracts prepared according to the procedure of Fuller et al.[329] At low KCl concentrations, wild-type plasmids were utilized as templates more efficiently than those purified from *dam*$^-$ strains. However, at moderate KCl concentrations their template activity was equivalent. Unfortunately, the majority of these experiments were done in the absence of a *dnaA* complementing fraction.

Purification of the factors responsible for replicating *oriC* has been initiated.[316,324,339] This system, which utilizes purified proteins and partially purified fractions, has allowed the determination of a number of steps in the initiation reaction. Polyvinyl alcohol was found to stimulate catenation by a topoisomerase in the soluble extract. This topoisomerase, termed "catenase α", was composed of a 100- and a 34-kDa subunit. Fractionation of a soluble extract from a *dnaA*$^-$ strain, using as an assay reconstitution of *oriC* DNA replication in the presence of the *dnaA* protein, RNA polymerase, DNA gyrase, the primosomal proteins, and the holoenzyme, yielded two fractions on DEAE cellulose. One fraction that eluted from the column with 0.2 *M* NaCl was capable of restoring DNA synthesis; however, this was not dependent upon either the *dnaA* protein or *oriC*. A combination of both fractions (the 0.2 *M* NaCl eluate and the flow-through) together yielded *dnaA*- and *oriC*-dependent replication.[324] One of the proteins responsible for this specificity is the large subunit of catenase α, identified to be topoisomerase I. The addition of antibody directed against topoisomerase I to the reconstituted system resulted in a switch to *dnaA*-independent DNA synthesis. However, it was suggested that additional factors, including protein HU (see below) were required for specificity.[324] In fact, recent data indicates that RNase H is a required specificity factor in *oriC* replication in vivo and in vitro as well.[340]

The small subunit of catenase α has been identified as exonuclease III.[341] This enzyme apparently enlarges nicks on RFII DNA, present as a contaminant in the RFI DNA preparations, to gaps, providing a preferred substrate for the catenation activity of topoisomerase I.[342,343] The requirement for polyvinyl alcohol may reflect the need for molecular crowding in such a catenation reaction.[344] It is not clear if catenase α, or only topoisomerase I, is the

component required for *oriC*-dependent DNA replication, since exonuclease III contaminates some of the other protein preparations required in the reaction.[341]

Another fraction, that eluted from DEAE cellulose at 0.25 *M* NaCl, was found to stimulate *oriC* replication three- to fivefold. The protein responsible for this stimulation was purified (based on its stimulatory activity) and identified as the HU protein.[324,339] Antibody directed against authentic HU protein inhibited the reconstituted *oriC* DNA replication system.[339] Maximal stimulation of *oriC* DNA replication occurred at a level of 40 dimers of HU protein per template DNA circle. This level is one tenth the level of HU protein that is required for maximal stimulation of transcription[345] or condensation of double-stranded DNA into a nucleosome-type structure.[346] Interestingly, in the presence of topoisomerase I these higher levels of HU protein will confer specificity (i.e., *dnaA*- and *oriC*-dependent replication) upon the reconstituted *oriC* DNA replication system.[324] This suggests that one mechanism of generating specificity in the *oriC* system may be the assembly of the DNA into some form of a nucleosome-type complex — a structure that would more closely resemble the template in the cell.

The kinetics of DNA synthesis in the reconstituted system showed a distinct lag that could be eliminated by preincubation of the template with all the protein components in the absence of dNTPs.[316,324] Omission of either DNA gyrase or RNA polymerase from the preincubation restored the full lag period, indicating that these proteins acted very early during *oriC* DNA replication. Omission of the *dnaA* protein from the preincubation resulted in a reduction in the lag period, indicating that this protein acted early in the reactions, but perhaps after the action of DNA gyrase and RNA polymerase. Fuller et al.[324] have suggested that initiation of replication at *oriC* may occur in the following steps: (1) template preparation (requiring DNA gyrase, topoisomerase I and the HU protein); (2) transcription and priming (requiring RNA polymerase and the *dnaA* protein); (3) primer processing (perhaps requiring RNase H, or perhaps requiring some, as yet, unidentified factor). The requirement for other proteins is clear since *oriC*-dependent DNA replication in the presence of RNA polymerase, DNA gyrase, topoisomerase I, the HU protein, SSB, the primosomal proteins, the holoenzyme, and the *dnaA* protein, still required the presence of partially purified fractions.[316,324]

The involvement of DNA gyrase in some early step during *oriC*-dependent DNA replication is supported by recent studies in which a strong, somewhat unique, DNA gyrase binding site has been identified by electron microscopy at position 253 ± 23 within the *oriC* sequence.[347] DNA gyrase binding at this site is not dependent upon either ATP or supercoiled DNA, suggesting that a specific sequence, rather than a structure, is being recognized. Binding is preferentially inhibited by oxolinic acid, in contrast to binding at other sites where oxolinic acids encourages double-stranded cleavage of the site by the bound gyrase molecule. In addition, DNA gyrase from *B. subtilis* will not bind specifically at this site. These data led the authors[347] to suggest that the DNA gyrase bound specifically in this manner may be required for some step in the initiation of DNA replication at *oriC*. It is interesting to note that two *dnaA* protein recognition sequences are present as an inverted repeat within the fifty nucleotides centered about position 250. This may provide the physical basis for the proposed interaction between DNA gyrase and the *dnaA* protein.

It is also possible that one of the partially purified fractions required for *oriC* replication in vitro[316,324] contains the B′ single-stranded DNA binding protein which can be isolated from membranes, that was identified by Jacq and Kohiyama.[348] Jacq et al.[349] have recently shown that this protein binds to two sites at *oriC*, corresponding to the counterclockwise strand at positions 38 to 165 and the clockwise strand at positions 472 to 488. The clockwise binding site is the additional sequence that Messer et al.[303] proposed was required for bidirectional replication from *oriC*. One of the four proposed *dnaA* binding sites in *oriC*, at position 88-80 on the counterclockwise strand, is included within the counterclockwise binding site of protein B′. This overlap may facilitate an interaction between protein B′ and

the *dnaA* protein. If such an interaction occurs, it may provide a means for coupling initiation of chromosomal replication to processes involved in membrane growth. If this were the case, however, one might have expected it to score genetically as a *par* region, which it does not do. The function of these membrane binding sites remains to be clarified.

## VII. LAMBDA dv

The replication of phage λ has been studied in detail. Replication, which requires the λ O and P proteins, the host RNA polymerase, and the products of the *dnaG, dnaB, dnaZ, dnaE, dnaJ, dnaK, grpP* and *grpE* genes,[350] begins bidirectionally in a circular molecule and proceeds progressively through a form of RF → RF replication to a rolling-circle mechanism.[351] Interactions between the λ P protein and the *dnaB, dnaK, dnaJ, grpE*, and *grpD* proteins, have been inferred from genetic data,[352] and in some cases demonstrated directly.[353] The minimal λ *ori* sequence lies within the λ O gene, and it has been demonstrated that the 32-kDa λ O protein binds to four 19 bp direct repeats within this *ori* sequence.[354]

Since λ DNA is a large duplex molecule, soluble systems were developed that replicated the circular λdv plasmid that utilizes the same replication machinery as the phage DNA.[355-357] Initiation in these systems has been mapped to the *ori* sequence within the O gene and is bidirectional.[356,358] The *ice* sequence, which was proposed to be the region required for transition from RNA to DNA on the nascent strand,[359-360] was not required in the crude system. These systems were characterized by their dependence upon λ O and P proteins, although there is not complete agreement upon the relative levels of these two proteins required. The λ $C_I$ repressor protein could inhibit λ dv replication in vitro.[358] Dependence upon some of the other *E. coli* replication proteins has also been reported.

The preparation of λ O protein in the laboratories of Klein[361] and McMacken[362] differ somewhat. The preparation from Klein's laboratory is composed of two species of slightly different size that share the same peptides after partial proteolysis. The preparation from McMacken's laboratory consists only of the larger species. Both species are equally active in binding to λ DNA, and in the λ single-stranded system (see below); however, the smaller species is twofold more active in λ *dv* replication system. The larger species in the only one made in vivo after λ infection;[414] thus, it is likely that the smaller species is produced by proteolysis.

A novel system for priming complementary strand synthesis using some of the proteins required for λ replication has been discovered.[358] This system apparently functions in a manner analogous to the primosome; however, it is nonspecific. It will prime any SSB-coated SS(c) DNA. The complete system was dependent upon the λ O and P proteins, the *dnaB, dnaJ,* and *dnaK* proteins, primase, SSB, and the holoenzyme. In the presence of ATP a prepriming complex could be isolated that required the λ O and P proteins, and the *dnaB, dnaJ*, and *dnaK* proteins. This complex was recognized by primase to form a psuedo-primosome that, uncoupled from elongation, could synthesize multiple primers on the DNA. How the role of the *dnaJ* and *dnaK* proteins in this system relates to their function, if any, during chromosomal initiation and elongation, remains to be resolved.

The 69-kDa *dnaK* protein has been purified to homogeneity and its characteristics studied.[363] It is a weak DNA independent ATPase that is relatively heat resistant. The λ P protein will inhibit this activity, whereas the λ O protein will stimulate it. Interestingly the *dnaK* protein, which is the major heat shock protein produced in *E. coli*,[364] and, in fact, appears to modulate the heat shock response, is a phosphotyrosine protein kinase that has an acidic pH optimium. It also has a 50% amino acid homology with the major heat shock protein, hsp 70, of *Drosophila*.[365] Currently, the only known substrate for the *dnaK* protein kinase is itself. The implications for the involvement in the control of DNA replication of this protein kinase replication protein have yet to be explored.

## VIII. BACTERIOPHAGE MU

Mu is a transposing bacteriophage whose characteristics of transposition have been well detailed.[366] Replication, at least in the case of Mu, is required for the transposition event. The fact that Mu is the most active transposon in *E. coli* suggested that its transposition event may be observable biochemically and has led both Higgins et al.[367] and Mizuuchi[368] to develop in vitro systems for Mu DNA replication. In order to ensure that they were observing DNA replication events coupled to transposition, Higgins et al.[367] used the cellophane disc system developed by Schaller et al.[9] This system measures DNA synthesis from preexisting forks. Initiation of the *E. coli* chromosomal replication does not occur. Mizuuchi[368] has developed a completely soluble system dependent upon exogenously added DNA.

Higgins et al.[367] prepared their extracts from exponentially growing cells that had been labelled with $^{3}$H-thymidine for 5 min just prior to lysis. DNA synthesis after lysis (on the cellophane disc) was allowed to proceed for 15 min in the presence of $^{32}$P-dNTPs. In general, an equivalent extent of DNA synthesis during both labeling steps was observed. When the crude extract was prepared from cells infected with Mu, a 30 to 45% increase in incorporation of radioactive precursor into DNA during the in vitro incubation ($^{32}$P), compared to uninfected cells, was observed. In addition, 30 to 50% of the nascent DNA in this case could hybridize to Mu sequences. The Mu specific sequences were distributed evenly in very large DNA and Okazaki fragments, suggesting that replication was semidiscontinuous. The Okazaki fragments hybridized preferentially to the Mu L strand, indicating that replication was left to right. In addition, since Mu contains 2 to 3 kbp of host sequences on its right-hand end, probing host Okazaki pieces with isolated Mu L and H strand indicated that there was no preferential orientation of Mu in the chromosome. These data demonstrate that when Mu is integrated into the chromosome it manages to direct unidirectional replication across itself.

Higgins et al.[369] have used density labeling to study the pattern of Mu DNA replication in this system. Density label (BrdUTP) was added during the in vitro stage of the incubation (with $^{32}$P-dNTPs), the DNA was gently sheared to an average size of 80 kbp and banded in CsCl. Extracts from uninfected cells synthesized DNA in vitro that banded largely at the hybrid density (H·L). This indicated that the length of DNA replicated by one fork in vitro exceeded 80 kbp. DNA synthesized in extracts from Mu infected cells in vitro also exhibited a peak at this density. However, a large amount of the DNA product banded between H·L and the fully light density (L·L). Only when this DNA was subjected to extensive shearing to yield smaller pieces would it band solely as H·L. Mu sequences were enriched in the hybrid density. Higgins et al.[369] suggested that the newly replicated Mu DNA was a combination of H·L plus L·L. Based on the relative sizes of the DNA labeled in the host (80 kbp), and the size of Mu (37 kbp), they proposed that replication forks traversing Mu were terminating within Mu sequences. This predicted, and it was demonstrated to be the case, that since replication across Mu proceeded preferentially left to right, the shortest new segments (i.e., the lightest and termed short-patch replication), would preferentially represent sequences from the right-hand end of Mu. Pretreatment of Mu-infected cells with chloramphenicol just before lysis inhibited this Mu-specific short patch replication. Since Mu transposition requires continuous synthesis of the Mu gene A protein,[370] Higgins et al.[369] concluded that this short patch replication was an indicator of Mu transposition in the cell.

Using extracts from cells infected with a Mu phage that carried a temperature sensitive A gene, Higgins and Olivera[371] have also gathered evidence indicating that A gene activity, that could first be accumulated in vivo by incubation of the cells at the nonpermissive temperature (after induction of Mu DNA replication by heat inactivation of the Mu gene C repressor protein), was then utilized for a burst of Mu specific DNA synthesis in vitro at the permissive temperature, thus confirming that initiation of Mu DNA synthesis was occurring in vitro.

Higgins et al.[372] have demonstrated that when experiments of the type described above were performed in the presence of NMN, even at very brief (5 min) labeling periods in vitro, the continuously synthesized Mu strand was very large and sedimented with the bulk of the chromosomal DNA prelabeled in vivo with $^3$H-thymidine. This suggested that Mu DNA replication was primed by chromosomal DNA and has led to the proposal that initiation of Mu replicative transposition occurred by the formation of a break in the left end of the Mu L strand, followed by attachment of the 5′ end of this strand to a chromosomal acceptor site. This series of events is presumably catalyzed by the Mu gene A protein. The structure generated is a replication fork and the 3′ end of the host DNA can serve as a primer for synthesis of the Mu continuous strand.

Mizuuchi[368] has developed an in vitro Mu DNA replication/transposition system that is more amenable to biochemical manipulation than the one described above. Extracts were prepared according to the method of Fuller et al.[329] Transposition donor DNA was in the form of pBR322 plasmids carrying the genetic ends of Mu DNA. λb221 was the recipient DNA. After incubation in the presence of the crude extracts to effect transposition, the λ DNA was packaged. The phage were plated on indicator bacteria to score total PFU. Ampicillin resistant colony formation scored transposition that resulted in the transduction of the pBR322 *bla* gene onto the λ phage DNA.

Transposition required that both ends of Mu DNA be present in the correct orientation on the donor DNA and was dependent upon extracts from uninfected cells as well as from cells carrying plasmids that overproduced the products of Mu genes A and B. Transposition was also dependent upon ATP and polyvinyl alcohol. It was not inhibited by chloramphenicol or rifamipicin, but was inhibited by novobiocin and NMN (in place of NAD). Transposition was also inhibited if the dNTPs were omitted and ddNTPs were added in their place. An analysis of the hybrid λb221-pBR322 plasmid DNA products from the ampicillin resistant colonies indicated that transposition was occurring by both simple insertions and co-integrate formation. A direct analysis of the DNA products after the transposition incubation confirmed these results, eliminating the possibility that any of the transposition events were occurring during the λ packaging incubation. The structure of the DNA products indicated that transposition must involve one cycle of semiconservative replication between the two Mu ends. This is in agreement with the findings of Higgins et al.[369]

## IX. BACTERIOPHAGE Φ29

*Bacillus subtilis* phage Φ29 replicates by a mechanism distinct from others extant in procaryotic cells. Initiation of DNA replication is catalyzed by a covalent complex formed between the product of the phage gene 3 and dAMP, the first nascent deoxynucleotide residue.[373,374] The gene 3 protein is also found covalently bound to the phage DNA purified from virions.[375] Formation of the initiation complex, that could be measured by the appearance of an α-$^{32}$P-dATP labelled gene 3 protein-$^{32}$P-dAMP complex on SDS gels, required protein linked Φ29 DNA and the product of the phage gene 2.[376,377] Gene 3 protein, purified from *E. coli* haboring a plasmid that overproduces it, will complement inactivated extracts prepared from Φ29 gene 3*ts* infected cells in the complex formation assay.[378]

Extracts prepared from Φ29 infected *B. subtilis* will support replication of exogenously added Φ29 DNA.[376,379] The products of phage genes 2, 3, 5, 6, and 17 are required. It is clear that complete reconstitution of the Φ29 replication system will require the purification of other proteins.[376] Recently, the gene 2 protein has been purified and identified as a DNA polymerase.[377,380] Gene 2 protein, purified from cells infected with Φ29 gene 2*ts* mutants, was thermolabile in its DNA polymerase, Φ29 DNA replication, and gene 3-dAMP complex forming activities.[377] The preferred template for the gene 2 DNA polymerase activity was poly (dA)·oligo(dT). Primed single-strands were significantly less active, perhaps indicating

that the enzyme was easily stopped by secondary structure in the DNA. The purified polymerase will elongate the gene 3-dAMP complex in the presence of Φ29 DNA; however, extracts prepared from cells infected with gene $3^-$, and gene $2^-$ double mutants, as well as extracts prepared from uninfected cells, stimulated the DNA replication reaction, confirming that there were additional host and viral coded factors required for the reactions.[377]

Garcia et al.[381] determined that there was a minimal size requirement for a DNA fragment from the terminus of Φ29 that would support gene 3 protein-dAMP complex formation. A 26-bp fragment from the left-hand end of Φ29 DNA would function whereas, a 10-bp fragment from the right-hand end would not. However, if the 10-bp fragment was blunt end ligated to fragments from λ DNA, activity could be recovered. Treatment of any DNA template with piperidine to remove the terminal protein resulted in a loss of activity. It is not clear if there is a specific DNA sequence requirement for the formation of the initiation complex.

The Φ29 system is remarkable for its similarity to the adenovirus DNA replication system[382] that initiates by an identical mechanism. There is no evidence, however, of processing of the Φ29 gene 3 protein, as there is for the 55-kDa adenovirus terminal protein. A question still unresolved, both in the case of Φ29 and adenovirus, is how these viruses acquired such a unique priming system. Judging from the requirement for a protein-linked DNA template, the detection of a similar priming system in the host, if it exists, would be difficult. However, since host factors are involved in both systems, it is reasonable to speculate that the also would interact with such a host priming enzyme. Affinity columns, of the type used by Formosa et al.[123] formed with some of these host factors, may be a means of detecting a hypothetical host protein priming system.

## X. COL El

The study of plasmid DNA replication has focused in recent years on the control of the initiation of DNA replication and on how this relates to plasmid copy number and the expression of incompatibility. These studies have revealed a number of control systems that are common to a collection of seemingly diverse plasmids. Both positive and negative control of DNA replication appears to be utilized, and on occasion, in the same plasmid system. Common themes include negative control of initiation of DNA replication by interference with either the production or processing of a primer or preprimer RNA transcript. In systems where a positively acting plasmid-encoded protein is required for replication, its production is generally negatively controlled by either another plasmid coded factor, or by itself. The positively acting plasmid-coded protein may not be rate limiting for replication, thus complicating the issue. The free concentration of these positively acting factors generally may be governed by the binding of the protein to a series of inverted or direct repeats on the plasmid DNA. Considering the rapid progress in this area, and the availability of in vitro plasmid DNA replication systems, it is likely that these various control loops will soon be completely detailed. A number of excellent reviews on these subjects will appear shortly[24-26] and they will not be detailed here. However, there have been several recent developments bearing on the replication of ColEl DNA that will be examined.

Mechanistically, the replication of ColEl DNA is probably the best understood of any closed circular double-stranded DNA. DNA replication is unidirectional, proceeds from a specific point on the H strand, requires no plasmid-encoded factors, and both DNA strands are synthesized essentially simultaneously.[383-385] The earliest replication intermediate detected is an approximately 400-nucleotide long nascent chain complementary to the plasmid H strand (the 6SL fragment).[386] Production of this nascent DNA chain is dependent upon DNA polymerase I. The 6SL fragment can be isolated as a D-looped replication intermediate.[387] Subsequent DNA synthesis requires the holoenzyme.[388]

The primer for L strand DNA synthesis (leading-strand synthesis) is formed from a transcript (the primer precursor, RNA II) synthesized by RNA polymerase and initiating 555 bp upstream of the origin. This transcript is specifically processed by RNase H at one of three consecutive nucleotide residues at the origin and is then elongated by DNA polymerase I.[389] Studies on a related plasmid (p15A), in which the 5′ and 3′ ends of the 3′ portion (RNA M) of the RNase H cleaved primer precursor RNA was mapped, indicated that when RNase H cleaved RNA II it eliminated, at most, 5 to 8 nucleotides of the continuous RNA II sequence.[390]

Control of the copy number of ColEl and expression of the plasmid-derived incompatibility functions are a result of the same event — regulation of the amount of RNA II that is used as a primer. This regulation involves both a protein and RNA species. RNA I, a transcript approximately 100 nucleotides long made off the L strand of the plasmid and complementary to the 5′ portion of RNA II, interferes with the RNase H catalyzed processing of RNA II.[391] Unprocessed RNA II molecules cannot be used as a primer (this view may have to be modified, see below). Interference of RNA II processing is through a direct RNA:RNA interaction between the two species. Elegant studies in a number of laboratories indicate that this interaction is the primary determinent of plasmid incompatibility and plasmid copy number (summarized in Reference 24).

RNA I can be folded into a tRNA-type structure with three stems and loops. Physical evidence for this structure has been obtained.[392,393] The 5′ end of RNA II can also, in theory, form a similar structure. The formation of the third (nearest to the 3′ end, structure III) stem and loop structure in RNA II precludes the formation of another, very large, stem and loop structure (structure IV), that is thought to be essential for primer processing. RNA I and RNA II are believed to interact through the complementary loops of the three stem and loop structures. This interaction presumably stabilizes structure III, thus inhibiting the formation of structure IV.[394] Moser and Campbell have demonstrated that proper functioning of this system is dependent upon the level of RNA I.[395]

The expression of incompatibility derives from the sequence of the loops of the three stem and loop structures. Thus, a change of one nucleotide in a loop will, depending upon the substitution, still allow the RNA I from that plasmid to interact with its own RNA II. However, an incoming wild-type plasmid may no longer be excluded because of the reduced interaction between the two different structures. A recent study indicates a good correlation between the calculated $\Delta G$ of the loop:loop interaction and the expression of incompatibility, as well as the copy number.[396] Mutations in the stem can give rise to temperature-sensitive copy number mutants and plasmids that express essentially no incompatibility. These mutations presumably affect the structure of the RNA I, preventing any interaction.

Using a plasmid containing two copies of the RNA I coding sequence, Campbell's laboratory[419] has identified copy number mutants that, because of the continued presence of a wild-type copy of RNA I (i.e., only one of the two copies was mutagenized), were dominant RNA I target mutations. All of those mutations that were sequenced fell within the loop of the central stem and loop structure of RNA II. This agrees with Lacatena and Cesareni[396] who, using a phasmid system, concluded that the central stem and loop structure was of primary importance in the expression of incompatibility.

Masukata and Tomizawa[397] have identified other regions of RNA II that are involved in RNA/DNA hybrid formation and primer processing. Their selection procedure was similar to that utilized by Lacatena and Cesareni.[396] Mutations (*pri*1, 2, 3, and 4) were isolated that effected the formation of the hybrid between RNA II and Col El DNA. These mapped at positions $-264$ to $-308$ (nucleotides upstream from the origin). Mutations (*spr*41 and 42) that suppressed the phenotype of this group of *pri* mutations could also be isolated that mapped to positions $-18$ and $-19$. These data imply that these two segments of RNA II interacted to influence the extent of RNA/DNA hybrid formation. A second type of *pri*

mutation (*pri*5) altered the site at which RNase H cleaved RNA II in the RNA/DNA hybird. The *pri*5 mutation changed the base pairing pattern at the base of a large stem-loop structure that could form in RNA II starting at position −9. In *pri*5 RNA II, the stem-loop structure started at position −14. RNase H cleavage of the *pri*5 RNA/DNA hybrid was at position −6, suggesting that the RNase H cleavage points were governed by the distance from the base of this stem-loop structure. Finally, a third type of mutated RNA II (*pri*6) was isolated that, while it formed RNA/DNA hybrid and was cleaved by RNase H in a normal fashion, could not be utilized as a primer by DNA polymerase I. *pri*6 RNA II had two changes at positions −186 and −188. It was suggested that this double mutation caused the region between −225 and −149 to assume an alternate structure that prevented access of DNA polymerase I to the primer terminus. A comparison of the RNase T1 cleavage patterns of *pri*6 and wild-type RNA IIs supported this argument. A suppressor of *pri*6 (*spr*61) restored the base pairing pattern of the wild-type stem-loop structure. These results indicate that certain structural requirements have to be satisfied in order for RNA II to form an active leading-strand RNA primer during ColEl DNA replication.

A second regulator of initiation of ColEl replication involves a small (63 amino acid) protein encoded by a region near the plasmid *nic* site.[398,399] Deletion of this region results in a four- to sixfold elevation of the copy number.[400] This protein, (*rop* gene product) has been shown to reduce the extent of expression of *lacZ* or *galK* expression when these genes were placed under the control of the RNA II promoter.[398,399] Som and Tomizawa[399] determined that a region on RNA II, roughly 50 nucleotides downstream from the 5′ end, was required in order to observe the effect of the *rop* gene product. Recent studies by Moser et al.[401] confirm that the *rop* gene product does not act as a classical repressor. It was found that the lethality, at the nonpermissive temperature toward the host, of a temperature-sensitive runaway replication derivative of pNTP1,pJN75, could be suppressed by the presence of the *rop* gene product. Thus, second-site mutations in pJN75 which rendered it refractory to this suppression (*nsr*, nonsuppressible by *rop* mutations) should define the *rop* gene product target site. Two *nsr* mutations were identified. These were within 10 nucleotides of each other and were roughly 100 nucleotides downstream of the 5′ end of RNA II. The authors suggested that the *rop* gene product acted by somehow enhancing the RNA I:RNA II interaction by making it more efficient. Since this explanation would incorporate both the *rop* gene product and RNA I into the same control loop, it would resolve the previous appearance of redundancy of the two control elements.

The coincidence of the *rop* protein and RNA I control loops has been established by Cesareni et al.[402] They found that the presence of a plasmid carrying only the *rop* gene, in cells where the gene for β-galactosidase (present on a phage) was controlled by the RNA II promoter, was not sufficient to modulate β-galactosidase synthesis. A second plasmid that carried the RNA I gene had to be introduced into the cell in order to inhibit β-galactosidase synthesis. A deletion in the RNA I promoter on the second plasmid restored insensitivity of β-galactosidase synthesis to the *rop* protein. Deletions of the RNA II-*lacZ* phage construct were used to define the *rop* protein target. They concluded that the target was at least in part more than 52 nucleotides downstream of the RNA II promoter, in agreement with Som and Tomizawa[399] and Moser et al.[401]

Lacatena et al.[403,404] have demonstrated inhibition of ColEl DNA replication by purified *rop* gene product in a crude system. This inhibition was also dependent upon the presence of RNA I. However, this replication system was inefficient and high levels (1000-fold excess) of the *rop* gene product had to be added in order to observe an effect. Thus, it is difficult to compare these results to those described above. Cesareni et al.[402] also used an in vitro transcription system to demonstrate that the *rop* protein, at protein to DNA ratios similar to those described above, induced premature termination of RNA II transcripts in the presence of RNA I — 20 to 30% of the RNA II transcripts terminated 220 nucleotides

downstream from the RNA II promoter. The remainder of the RNA II transcripts could not be used as a primer, suggesting that some of the specific RNA II structural requirements for active primer formation identified by Masukata and Tomizawa[397] may have been altered. Thus, it is clear that the *rop* protein-RNA I control loop is very complex and can respond to subtle alterations in both RNA I and RNA II.

The identification and position of pas-CH and pas-CL on ColEl DNA, and pas-BH and pas-BL on pBR322, led to the suggestion that the L strand pas served as the origin of lagging-strand DNA synthesis. The H strand pas was proposed to be the site at which continuous extension of the L strand by DNA polymerase I terminated and synthesis dependent upon the primosome and the holoenzyme began.[32] Alternatively, it could be used to catalyze initiation of DNA synthesis on the mobilized plasmid H strand after conjugal transfer.[190,191] Previous data suggested a role only for the *dnaB* protein during L strand synthesis, whereas H strand synthesis required the products of the *dnaB, dnaC,* and *dnaG* genes.[405]

An active role for pas-C(B)H in ColEl(pBR322) DNA replication was considered unlikely due to the isolation of deletion mutants of both plasmids which lacked the H strand site.[400,406] In fact, pBR322 derivatives have been constructed which lacked both pas sequences that were as stable as the wild type, but had a copy number two- to threefold lower.[407] It should be pointed out, though, that these plasmids also had the *rop* gene deleted. This normally results in a four- to sixfold elevation of the copy number. Thus, the reduction in copy number here should be more properly viewed as being in the 8 to 18-fold range, a significant decrease, suggesting that one, or both, of these sites are important in plasmid replication.

Support for this view comes from the recent observations that both leading- and lagging-strand ColE1 DNA synthesis was blocked at the nonpermissive temperature in strains carrying the temperature-sensitive *dnaG*3 or *dnaG*308 mutations.[408] While this conflicts with previous data indicating that the *dnaG*1011 mutation blocked only lagging-strand synthesis,[405] it is possible that the primases produced from these different alleles may be affected differently in their leading- and lagging-strand functions.

In addition, studies by Arai and colleagues,[420] using a crude in vitro pBR322 DNA replication system, indicated that at least the L strand site, and perhaps the H strand site, had a function in pBR322 DNA synthesis. Deletion of the L or H strand sites resulted in a ten- and threefold decrease, respectively, in DNA synthesis observed. Replicative activity could be restored by inserting pas-X on the L, but not the H strand, of the pBR322 DNA molecule deleted for pas-BL. Plasmids lacking pas-BL arrested replication after formation of the 6SL fragment. These data indicate that the L strand pas sequences in pBR322 and ColEl DNA are likely to be the plasmid lagging-strand origins of replication, and that the H strand site may serve some function during replication. The conflicting data regarding the synthesis of the L strand may be due to the existence of two possible modes of elongation after formation of the 6SL fragment, one dependent upon the primosome and the holoenzyme, and one dependent upon the holoenzyme alone. Resolution of this question awaits the reconstitution of pBR322 DNA synthesis with purified proteins.

Recent studies by Okazaki and colleagues[409,410] questions the role of RNase H (encoded by the *rrnh* gene) in ColEl replication. They isolated host mutations (*her*) which were capable of suppressing *ColEl r*eplication defective mutants (*cer*).[409] Using a phasmid system, suppression of *cer* mutants was scored as the replication of the phasmid as a plasmid. A number of *her* mutants were isolated in this manner and were shown to be *cer* allele specific. One *her* mutation, capable of suppressing *cer*6, a single base change at position −160 (relative to the origin, note that the *spr*61 mutation[397] is at position −161), mapped to the *rrnh* gene. In vitro measurements demonstrated that RNase H activity was reduced to 0 to 0.2% of wild-type levels in this strain. It was indicated that initiation of plasmid DNA replication was occurring at the legitimate ColEl *ori*. In addition, several independently isolated *rrnh*

mutations supported the replication of the *cer*6 mutant plasmids.[410] This intriguing observation awaits explanation. Since formation of the RNA/DNA hybrid between ColEl DNA and RNA II is an event that is independent of the presence of RNase H, it is possible that in the absence of RNase H the 3′ nonhybridized tail of the RNA II in the hybrid can be eliminated by some nonspecific RNA exonuclease. It would be of interest to study the behavior of the *pri* mutants[397] in these mutant strains.

The role of the *dnaA* gene product is another aspect of pBR322 DNA replication that should be reexamined in light of the recent demonstration of a *dnaA* protein recognition sequence on pBR322 DNA.[316] There had been a previous indication that at the nonpermissive temperature, ColEl replication shifted to a rolling-circle mode in *dnaA ts* strains.[411] Polaczek and Ciesla[412] now report that if rifampicin was added to *dnaA ts* strains carrying plasmid pBR322 one hour after a shift up to the nonpermissive temperature, pBR322 DNA replication was inhibited. However, 2 hr later it resumed. At the permissive temperature, no resumption was observed. The intact *dnaA* protein was apparently not required since the effect was also observed with amber *dnaA* mutations. It is not clear if these observations reflect a process normally occurring during pBR322 DNA replication. One possibility is that the *dnaA* protein and RNA polymerase act, in some fashion, to unwind the plasmid DNA enough that a helicase could enter, enabling further unwinding and exposure of a pas. Once in a single-stranded form the pas could function as the origin of DNA replication.

These results should remind investigators using hybrid plasmids formed between pBR322 DNA and other origin sequences that replication of these chimeric molecules in a *pol A*$^-$ background is not necessarily an indicator of proper functioning of the *ori* sequence in question. There are apparently multiple ways in which pBR322 DNA can manage to survive, particularly if pas-BL is exposed in single-stranded form because of transcriptional activation or some protein-induced unwinding event.

## XI. CONCLUSION

A considerable number of person-years of sustained effort by the members of a number of laboratories has resulted in the isolation, in highly purified form, of nearly 50 proteins involved in DNA replication. The availability of these proteins has allowed the reconstruction and detailed analysis of many of the partial reactions which occur duing DNA replication.

Current trends are toward the study of some of the more subtle aspects of DNA replication. Is leading- and lagging-strand DNA synthesis coupled? What influences the progress of the replication fork? What structure does an origin of replication assume and what are the signals in the DNA which are required to effect initiation? How are the myriad interactions between the replication proteins controlled?

It is likely that future studies in prokaryotes will yield an understanding of the control of the initiation of DNA replication, a better picture of how the native protein-DNA complex in the cell participates in DNA replication, the effect of the topology of the DNA on its replication, the mechanism of coupling of DNA replication to the cell cycle, the termination of DNA replication and the mechanism of segregation of daughter chromosomes, and insight into the mechanism of chromosome sorting.

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## Errata

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### Thermodynamics of Hapten-Antibody Interactions

Equation 4 on page 137 should have appeared as follows:

$$Ki = \frac{n-i+1}{i} K$$