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INITIATION OF DNA SYNTHESIS IN *ESCHERICHIA COLI*[1]

Jun-ichi Tomizawa and Gerald Selzer

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

CONTENTS

PERSPECTIVES AND SUMMARY

Because DNA contains the genetic information of the cell, its replication is one of the most important of the events that occur as the cell grows. The process that carries out this replication can be conveniently subdivided into three stages: initiation, continuation, and termination. In this review, we describe recent progress in understanding the mechanisms by which DNA synthesis can initiate in *Escherichia coli.* The stages that follow initiation

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are not discussed except when they provide information about initiation itself.

Many approaches have been taken to the study of DNA synthesis. These include physical and chemical studies on the structure of DNA, biochemical studies on enzymatic mechanisms of DNA synthesis (1–9), and genetic studies of the functions required for DNA replication and its regulation (10). For a long time these studies were performed more or less separately, and it is only recently that a comprehensive understanding of DNA replication has begun to emerge.

Both DNA and RNA are synthesized by the ordered polymerization of nucleotides in a sequence complementary to that of the template strand. While RNA polymerases can initiate polymerization at specific nucleotide sequences, no known DNA polymerase can copy a template unless presented with the 3'-hydroxyl terminus of a polynucleotide primer that is based-paired to a single strand of the DNA template. Thus it is the process of primer formation that determines the site where the initiation of DNA synthesis occurs.

Considerable information about primer formation has been gained through the study of in vitro DNA synthesis using the single-stranded DNAs of small phages like fd and ϕX174 as templates. These studies have shown that both RNA polymerase and the *dnaG* protein can make primers for DNA synthesis. Depending on the kind of template DNA, one or the other enzyme is used.

Initiation of DNA synthesis on double-stranded DNA requires separation of the DNA strands in addition to the formation of primer. One way to accomplish this is by making use of the local strand separation that occurs during transcription by RNA polymerase. In this case, the transcript may itself serve as primer. Another mechanism involves the nicking of one strand to expose a 3'-hydroxyl group. The strands can be separated at the nick by an enzyme, and the 3'-hydroxyl group can then serve as primer.

As double-stranded DNA replicates, growth of one of the two progeny strands is usually occurring at the 5' end of the strand, even though polymerases are only known to add nucleotides to 3' ends. It is now clear that this growth is accomplished through the addition of small DNA fragments to the 5' end. Each of these fragments is synthesized shortly before it joins to the strand.

Thus there are two roles for primer synthesis during replication. One of these is the initiation of replication of the genome, while the other is the initiation of synthesis of each of the small fragments made during elongation of the progeny strands. Despite this difference in function, the two kinds of primer synthesis could be carried out by the same mechanism.

The elucidation of the nucleotide sequences of regions where DNA synthesis initiates is an area in which major progress has recently been made.

Most of the known sequences can be arranged into extensive secondary structures. As yet, however, there is practically nothing known about the significance of these structures. Their role in the protein-nucleic acid interactions that occur during the initiation of DNA synthesis is an interesting problem that is now accessible.

Characterization of the proteins that participate in DNA replication and the precise determination of the location where DNA replication initiates became possible only after the development of in vitro systems that carry out DNA replication. For this reason, our discussion of the basic mechanism of initiation depends heavily on information obtained from in vitro experiments. Since it is difficult to perform experiments with a large genetic element like the chromosome of *E. coli,* much of this information has been gained by use of the DNAs of small viruses and plasmids. Recently, small plasmids that appear to contain the origins of replication of large bacterial and viral genetic elements have been isolated. Future in vitro studies of the replication of such plasmids should clarify the biochemical mechanisms involved in initiation of replication at these origins.

DNA SYNTHESIS ON SINGLE-STRANDED CIRCULAR DNA

A number of small phages of *E. coli* are known to have circular single-stranded DNA genomes of similar size (about 6000 bases). Although these phages can be divided into two groups (filamentous and isometric) on the basis of morphological differences, their genomes replicate by similar mechanisms. In the first stage of this replication, the viral DNA [(+) strand] is converted to a circular duplex called an RF molecule. The process, which is sometimes called SS → RF synthesis, involves synthesis of the complementary strand [(–) strand] in a reaction that requires no phage-specified protein. The next stage of DNA synthesis (RF → RF synthesis) begins when a phage-encoded protein breaks the RF molecule at a specific position on its (+) strand. The new (+) strand is then synthesized by extension of the 3′ end at the break by a mechanism involving strand displacement. Completion of synthesis yields a new circular (+) strand. The displaced (+) strand may serve as template for synthesis of the (–) strand of a new RF molecule which could begin while (+) strand synthesis is still in progress. The third stage of replication (RF → SS synthesis) occurs at later times in infection when synthesis of (–) strands on the displaced (+) strand is blocked by viral proteins that encapsidate the (+) strand.

Synthesis of (–) strands on single-stranded viral DNA initiates with the formation of a primer synthesized either by RNA polymerase (fd and other filamentous phage) or by the *dnaG* protein (ϕX174 and other isometric phage). Subsequent DNA synthesis is carried out by the DNA elongation

components. In most in vitro experiments, elongation of the primer has been carried out by DNA polymerase III in combination with *dnaZ* protein, and the DNA elongation factors I and III (8, 11), or else by a protein complex that contains the polymerase and its accessory factors (DNA polymerase III holoenzyme) (12, 13). However, elongation can also be performed by DNA polymerase I or DNA polymerase II. Since recent reviews of the subject are available (7, 8), the DNA elongation reaction is not discussed here.

In the section that follows, the in vitro synthesis of complementary strands is examined in some detail. Following this, the mechanisms of RF → RF and RF → SS synthesis are described. [Reviews: in vivo, (14–18); in vitro, (6, 8).]

Priming by RNA Polymerase

The synthesis of DNA on (+) strands of filamentous phage M13 (or fd) in cell extracts initiates at a specific region of the template (19). In addition to dNTPs, the synthesis requires all four rNTPs (20, 21), and is inhibited by rifampicin (21, 22), a specific inhibitor of *E. coli* DNA-dependent RNA polymerase. The products of synthesis are (–) strands of nearly full length.

The synthesis of (–) strands can also be carried out with purified proteins, namely DNA-dependent RNA polymerase, DNA binding protein I [DBP, (23)], and the DNA elongation components (24). It has been proposed that the primer is synthesized at a unique position that is not covered with DBP and is therefore accessible to the RNA polymerase (6, 24). According to this model, a duplex hairpin structure in the (+) strand blocks binding of DBP to the region where the primer is formed.

The structure of fd DNA in the region where RNA polymerase binds has been characterized by the following experiments. Exhaustive DNase digestion of fd DNA in the presence of RNA polymerase and DBP yielded protected fragments of about 120 nucleotides in length (25). Pyrimidine tract analysis of these RNA polymerase-protected fragments showed that they come from a region known to contain the origin of synthesis of the (–) strand (26). Examination of the nucleotide sequence of this region further showed that the protected fragment could form two separate hairpin structures. As described below, one of the hairpin structures includes the DNA that specifies the primer of (–) strand synthesis. The other hairpin has been considered unimportant for replication because its alteration by the insertion of foreign DNA does not abolish the infectivity of the DNA (27). However, the site of this insertion is near the loop of the hairpin; thus the importance of the stem region has not been determined.

When fd DNA is incubated with RNA polymerase, DBP, and rNTPs, a transcript of about 30 nucleotides in length is made. The results of

restriction enzyme analysis of DNA fragments formed by extension of the RNA transcript by DNA polymerase I have been combined with RNA fingerprint analysis of the primer itself to provide the exact location of the RNA on the nucleotide sequence of fd DNA (28). The transcription initiates with ATP at a dTMP that is not base-paired, but is located near the stem of the hairpin structure containing the primer sequence. There is no nearby heptanucleotide sequence of the type usually found in promoter sequences (29, 30). The transcription proceeds toward the loop of the hairpin structure. With the progress of transcription, DBP may complex the opposition strand of the hairpin as the structure melts. Since transcription terminates anywhere within a region of several nucleotides, it appears possible that termination results when RNA polymerase encounters DBP in the region of the melted hairpin structure (28).

The nucleotide sequences of the region containing the origin of (–) strand synthesis of M13 (31) and f1 (32) DNAs have also been determined. These have extensive homology with the fd sequence and, in particular, have almost completely conserved hairpin structures.

As discussed in the next section, ϕX (–) strand synthesis in cell extracts does not use RNA polymerase for its primer synthesis. Nonetheless, ϕX (–) strands can be synthesized by the same mixture of RNA polymerase, DBP, and DNA elongation proteins used for fd (–) strand synthesis (33, 34). If the purified RNA polymerase is replaced by a crude enzyme preparation with RNA polymerase activity, ϕX (–) strand DNA can no longer be made, although M13 DNA is still synthesized (33). Starting with a cell extract, three proteins have been obtained that specifically inhibit ϕX DNA synthesis when added together to the enzyme mixture containing purified RNA polymerase (34, 35). One of these has been identified as ribonuclease H (RNase H); the other two are referred to as discriminatory factors α and β. Although the exact role of these proteins in the discriminatory effect is unknown, the involvement of RNase H suggests that the effect may result from the degradation of ϕX transcripts which have potential priming function.

Priming by dnaG Protein

With DNA of the isometric phages (ϕX174, G4, ST-1, etc), the first step in (–) strand synthesis involves formation of a primer by the *dnaG* protein. Remarkably, the various isometric phages differ with respect to the number of factors, besides the *dnaG* protein, that they use for primer formation.

Synthesis of (–) strands of G4 (or ST-1) can be demonstrated with purified *dnaG* protein, DBP, and the DNA elongation components (36–41). The process of DNA synthesis by these proteins consists of several discrete steps (8, 39–41). In the first step, DBP covers the template DNA and the

dnaG protein then binds. No nucleotide cofactor is required in the process. Once formed, the DNA-protein complex can be isolated by gel filtration (41–43). The complex contains approximately one molecule of *dnaG* protein for each molecule of template DNA even when excess protein is present in the incubation. Binding of the *dnaG* protein in the presence of DBP is specific for G4 (or ST-1) DNA since no stable complex is formed with fd or ϕX DNA (42, 43).

The exact NTP requirement for primer synthesis by *dnaG* protein has been a subject of some controversy (36, 40). A partial resolution was provided by the discovery that the *dnaG* protein can synthesize oligonucleotides that consist of ribo-, deoxyribo-, or mixed ribo- and deoxyribonucleotides (41, 44, 45). To some extent, ribonucleotides and deoxyribonucleotides are used interchangeably for primer formation. Efficient G4 primer synthesis can occur in the absence of all rNTPs but only if ADP is present (42, 43). For ST-1 primer synthesis, even ADP is not required. In its absence, the ST-1 primer initiates with dATP at the 5' end (42, 43). Irrespective of its chemical composition, the oligonucleotide that can be elongated by the DNA elongation components is called a primer, and the *dnaG* protein is called primase (46).

When all four rNTPs are present, an oligoribonucleotide of 25–28 residues is synthesized on G4 DNA by the *dnaG* protein and DBP. The base sequence of the oligonucleotide has been determined (47). Regardless of its exact length, the product can serve as a primer for DNA synthesis (47). Recently it has been shown that the size of the oligonucleotide products is affected by the conditions of the reaction (48). When a low salt concentration (50 mM NaCl) and a high temperature (39°) are used, most of the oligonucleotides made on G4, ST-1, ϕK, and α3 DNAs have a length of 28 bases. When the salt concentration is raised (250 mM) and the temperature lowered (15°), the length decreases to 14 bases with G4 and 11 bases with the other DNAs.

The nucleotide sequence of the region of DNA where the synthesis of (–) strands initiates has been determined for G4 (49, 50) and for ST-1, ϕK, and α3 (51). Within this region (about 300 nucleotides), the sequences of ST-1, ϕK, and α3 are similar to each other, while differing significantly from that of G4. There are two stretches, one 42, the other 45 bases in length, that are quite similar in all four phages. These two stretches are separated by 13 bases whose sequence differs among the various DNAs (51).

In each case, the region that specifies the primer lies within one of the conserved stretches. The sequence of this region in ST-1 is identical to that of ϕK and α3, but different at 5 positions from that of G4. Despite the difference, either sequence can be arranged in a similar hairpin structure with a stem of 8 base pairs and a loop of 5 bases.

Because of the effect of salt and temperature noted above, it appears that the exact size of the product made by the *dnaG* protein may be determined by the stability of this hairpin structure. Comparison of the various sequences does not suggest a sequence or structure, other than that of the hairpin itself, which could play a specific role in the interaction of the DNA with the *dnaG* protein.

Priming by dnaG Protein in Conjunction with Other Proteins

Synthesis of ϕX (–) strand requires at least 11 proteins, only 6 of which are needed for G4 (–) strand synthesis. All of the additional proteins act before or during synthesis of the primer which is again carried out by the *dnaG* protein. The additional proteins include the *dnaB* and *dnaC* proteins, factors X, Y, and Z (8, 43, 52). These three factors probably correspond to proteins i, n', and n, respectively, which have been independently isolated and named (13, 53). In the absence of any of these components, the *dnaG* protein cannot catalyze primer formation with ϕX DNA as template. However, primer synthesis can occur in the absence of the proteins required for DNA elongation (43, 54).

The sequence of events during priming can be summarized briefly as follows. First, DBP covers the DNA and one or several molecules of factor Y (protein n') and Z (protein n) bind as well. Then the *dnaB* protein is transferred to the protein-covered DNA in a reaction that requires the *dnaC* protein, factor X (protein i), and ATP. The net result of these reactions is formation of a DNA-protein complex containing the *dnaB* protein, factors Y and Z (proteins n' and n), and DBP (8, 43, 54). The *dnaG* protein now binds and begins synthesis.

The reactions that occur after binding of the *dnaB* protein are as yet unclear. However, some insight into the mechanism may be provided by the following results obtained through the use of antibodies (55, 56). Addition of antibody directed against the i, n, or *dnaB* proteins inhibits *dnaG*-dependent transcription and also DNA synthesis by the reconstituted system for ϕX (–) strand synthesis. Once the DNA-protein complexes containing the *dnaB* protein are formed, transcription is inhibited by the anti-*dnaB* antibody, but not by the others. If primer has been made, none of these antibodies inhibits DNA synthesis. These results suggest that proteins i and n function before binding of the *dnaB* protein to the DNA, and that once primer is made, even the *dnaB* protein is not needed for subsequent DNA synthesis.

One drawback to this kind of experiment is the possibility that a particular antibody may be ineffective because the site it recognizes on a protein is masked in a complex. Another is the difficulty in performing precise

kinetic analysis. Firmer conclusions about the roles of the various components probably require a more direct analysis of the proteins present in the intermediate complexes.

Unlike transcription of G4 DNA, where a single unique primer is synthesized, a yield of six to eight oligonucleotides per template can be obtained when ϕX DNA is used (57). These oligonucleotides vary in length from 16–50 residues and appear to be of heterogeneous sequence. Even under conditions where a yield of one or less oligonucleotide per template is obtained, no strongly preferred site of transcription is evident (57). From the available data, no conclusion can be drawn about the regions transcribed or about which transcripts actually serve as primers.

Recently it has been shown that protein n' (factor Y) binds preferentially to the single-stranded *Hae*III-Z1 fragment, which contains 25% of the ϕX genome (13). Thus it appears possible that the *dnaB* protein, guided by protein n', binds at a unique site on ϕX DNA. To reconcile the heterogeneity of the transcripts with the assumption that the *dnaG* protein binds and initiates primer synthesis only where the *dnaB* protein is present, it has been proposed that the *dnaB* protein, once bound, migrates on the template (56, 57).

As is the case for G4 (or ST-1) primer synthesis, the *dnaG* protein can synthesize ribo-, deoxyribo-, and mixed ribo- and deoxyribooligonucleotides on ϕX DNA (43, 57). It appears that similar numbers of products are made with either rNTPs or dNTPs as substrate, but that the probability of termination is increased by the presence of dNTPs. Possibly because of the variety of products, the nucleotide requirements for effective ϕX primer synthesis (57, 58) have been far less simple to establish. Except for an absolute requirement for ATP (21, 43), the efficiency with which dNTPs substitute for the corresponding rNTPs is still an open question.

With the obvious exception of ϕX, the nucleotide sequences of the isometric phages are very similar in the region used as the origin of (–) strand synthesis. This also appears to be true for the analogous region of the filamentous phages. Since in all these cases the regions are intercistronic, it seems likely that the sequence is conserved primarily because of the necessity of a specific site for initiation of DNA synthesis. Larger genomes, such as the *E. coli* chromosome, need to repeat the initiation events required for discontinuous synthesis many times during a round of replication. Use of such a site-specific mechanism, which would require many copies of the same long sequence to be present in the genome, could lead to disintegration of the chromosome through recombination. From this viewpoint, the mechanism employed for ϕX (–) strand synthesis is a more suitable method for replication of large genomes. For the moment, the existence of the *dnaG* reaction observed with G4 remains a puzzle.

DNA SYNTHESIS ON DOUBLE-STRANDED DNA

DNA Synthesis from a 3'-hydroxyl End at a Strand Break

In some cellular processes, DNA synthesis initiates at a pre-existing 3' hydroxyl end of a DNA strand. Such processes include the DNA synthesis which seals gaps formed during replication, genetic recombination, and excision repair. In other cases, a 3'-hydroxyl end is created by specific nicking of duplex DNA and then used as a primer for initiation of DNA synthesis. The synthesis of (+) strands on RF molecules of the small single-stranded DNA phages provides the best-studied example of this kind of initiation (59). In the discussion that follows, DNA synthesis on ϕX RF molecules is described in some detail, and then a briefer description of other systems is given.

In vivo studies have shown that DNA synthesis on ϕX RF molecules requires the phage gene *A* protein (60). This protein breaks the (+) strand of an RF molecule (61, 62) in the *Hind*II-R3 segment that includes a portion of the gene *A* itself (63). The protein remains attached covalently to the 5' end of the broken strand (64, 65). Both supercoiled DNA and circular (+) strand DNA are susceptible to the action of the protein, but relaxed closed-circular, open-circular, and linear duplex DNAs are not (64, 65). The nucleotide sequence at the 3' terminus of the cleaved (+) strand has been determined (66). The terminus is located at a unique position in the known nucleotide sequence of ϕX DNA (67). That the action of the gene *A* protein is actually the first step in initiation of DNA synthesis has been suggested by an in vivo experiment which shows that the *Hind*II-R3 segment contains the region synthesized first during RF replication (68).

DNA synthesis on RF molecules is observed in extracts from uninfected bacteria that are supplemented with the gene *A* protein (69–71). These extracts are inactive when prepared from a mutant strain (*rep*$^-$) unable to support ϕX replication in vivo (72). A set of proteins that can carry out DNA synthesis on RF molecules has been purified (69–71). When supplied with RF molecules, the mixture of gene *A* protein, *rep* protein, DBP, and DNA polymerase III holoenzyme produces (+) strand circles in numbers that far exceed the number of input template molecules (69). Following fixation with formaldehyde and glutaraldehyde, replicating molecules from this purified enzyme system have been examined in the electron microscope and found to have a single-stranded loop attached to a double-stranded circle at a single point ("looped" rolling circle) (73, 74). This observation suggests that the 5' end moves along the circular molecule with the replication fork. When a round of synthesis is completed, a circular (+) strand could be produced through a transfer reaction catalyzed by the gene *A* protein (73).

Although the strands of an RF molecule separate as DNA synthesis proceeds, DNA synthesis is not obligatory for strand separation. In the absence of the DNA polymerase III holoenzyme, the RF molecule is separated into circular and linear strands by a mixture of the gene *A* protein, *rep* protein, and DBP (75–77). Recently, it has been shown that even the gene *A* protein is not required for strand separation (78). Together with DBP, the *rep* protein can separate the strands of DNA that contains gaps, but not the strands of nicked DNA. Since the addition of DBP before the *rep* protein prevents the reaction, the single-stranded region is probably required for binding of the *rep* protein (78). Two molecules of ATP are hydrolyzed for every base pair melted, whether or not the strand separation is coupled to DNA synthesis (77, 78).

Although the in vitro system which contains only the gene *A* protein, *rep* protein, DBP, and the DNA polymerase III holoenzyme produces complete circular (+) strands, other results suggest that additional *E. coli* proteins may participate in (+) strand synthesis in vivo. For example, a partially purified bacterial extract supplemented with the gene *A* protein cannot support synthesis of (+) strand from RF molecules if its *dnaB, C,* or *G* protein has been inactivated (79). The synthesis of (+) strands in vivo may also require these same gene products (80). Other experiments suggest that both strands of the ϕX RF molecule are replicated in vivo by a discontinuous mechanism (81), a finding hard to reconcile with the simple model presented above. These various results indicate to us that there may be factors or reaction conditions that determine the exact reaction mechanism to be used for in vivo (+) strand synthesis. Changes in these conditions or loss of the factors during purification of the proteins used for in vitro synthesis may permit the occurrence of reactions that normally play a minor role in synthesis in the cell.

The site of the origin used for synthesis of the G4 (+) strand has been deduced from the location of the gaps in open-circular molecules that accumulate at late times in infection (82, 83), as well as from the position of the end of the tail in rolling circle molecules (84). This site is located about 40% of unit length away from the origin of (–) strand synthesis. When the nucleotide sequence of the region containing the origin of G4 (+) strand synthesis is compared with that around the site cleaved by the gene *A* protein in ϕX DNA, an identical 30 nucleotide sequence can be seen in both DNAs (48). This is the longest stretch of sequence homology in these two DNAs (85). These results, coupled with the observation that G4 is unable to replicate in *rep*$^-$ bacteria (86), suggest that G4 also makes use of a site-specific endonuclease to initiate RF $\rightarrow$ RF replication. There is, as yet, no direct evidence for such a nuclease.

Despite the apparent similarity of the ϕX and G4 initiation mechanisms, RF molecules of G4 are not found in rolling-circle structures during the early phase of infection. Instead, displacement loop (D- loop) structures similar to those seen in replicating mitochondrial DNA (87) are observed (88). As mentioned above, circles with a single-stranded tail (rolling circles) are found at later times (85).

Replication of RF molecules of the filamentous phages (M13, fd, and f1) depends on the phage gene *II* protein (89, 90) and the host *rep* protein (91). The gene *II* protein breaks the (+) strand at a specific site (92). In contrast to the ϕX gene *A* protein, the gene *II* protein does not attach covalently to the cleaved strand (93). The cleavage site is separated from the site where synthesis of the primer for (–) strand synthesis initiates by 24 nucleotides (92). The inference that the cleavage site is the origin of (+) strand synthesis is consistent with the observation that, in vivo, the origins of synthesis of the (+) and (–) strands of f1 DNA are located close to one another (94).

As mentioned above, the nucleotide sequence in the origin region is very similar in these filamentous phages (27, 31, 32). In this region there is a six-base homology with the sequence near the origin used for ϕX (+) strand synthesis. Remarkably, the gene *II* protein cleaves fd DNA within this short homology while the ϕX gene *A* protein cuts ϕX DNA just 3 bases outside it.

In vivo RF $\rightarrow$ RF replication of M13 requires the *dnaG* (95, 96) and *dnaA* (97) proteins. However, these proteins are not required during either SS $\rightarrow$ RF or RF $\rightarrow$ SS synthesis in vivo. Thus there appears to be a requirement for certain proteins that is unique to the mechanism that produces progeny RF. The availability of DBP or of phage capsid proteins may determine the exact mechanism to be employed and thus the protein requirement.

Synthesis of (+) strands on fd RF molecules by the gene *II* protein, DBP and DNA polymerase III holoenzyme has been reported (93). It is possible that the *rep* protein, which is required for in vivo replication of the RF molecules, was present in the preparation of gene *II* protein that was used.

Initiation of DNA synthesis by the breakage of a strand is not limited to these small DNA phages. In vivo experiments show that replication of phage P2 DNA requires the P2 gene *A* protein (98) which helps catalyze a strand-specific break in the region where replication initiates (99, 100). As with that of the small phage, replication of P2 requires the host *rep* protein (101).

Nicking and extension of DNA strands may also occur during phage T7 DNA synthesis. In the absence of rNTPs, the T7 DNA polymerase and T7 gene 4 protein can catalyze DNA synthesis from preexisting nicks (102–

104). Normally, no synthesis occurs when these proteins are incubated with intact T7 DNA. However, if an *E. coli* protein, named initiation protein, is also included in the incubation, synthesis initiates from nicks introduced at three specific sites on the light strand (104). Alone, none of these proteins exhibit any nucleolytic activity. Approximately 70% of the synthesis initiates at a site about 18% from the left end of the linear DNA and proceeds rightward to give a product in which the newly synthesized DNA is attached covalently to the parental light strand. Although this site of initiation is close to the site of initiation of in vivo replication, if not identical to it, the synthesis does not give rise to the replication loop intermediate observed in vivo (2).

Recently a hybrid plasmid containing the in vivo origin of T7 has been constructed and shown to replicate in extracts of T7-infected bacteria (105). As yet, little is known either about the proteins required for this replication, or about the DNA produced. Future study of the system should prove useful in understanding the T7 initiation process.

Processing of Primer RNA and Use of Two Initiation Mechanisms

These two phenomena are observed during in vitro replication of ColE1 DNA. Although unrelated, they are presented together in this section. Recent reviews of ColE1 DNA replication are available (8, 106, 107, 107a).

Synthesis of ColE1 DNA can be carried out in vitro using extracts of *E. coli* cells (108). Extracts of bacteria that do not carry ColE1 are effective, even when de novo protein synthesis is blocked during the incubation (109). This result indicates that proteins encoded by the plasmid are not required for its replication in vitro. That this is also the case in vivo has been shown by the replication of ColE1-phage hybrids following infection of chloramphenicol-treated cells (110, 111).

The major products of the in vitro reaction are completely replicated molecules and other molecules (early replicative intermediates) having a small replication loop in a unique region. Such loops contain newly synthesized small DNA fragments (6S fragments) (108, 112, 113). A specific fragment of L-strand DNA (6S L fragment) is the first product of synthesis (114).

Synthesis of the 6S fragments in cell extracts can be inhibited by rifampicin (108), novobiocin (115), or nalidixic acid (116), which indicates the involvement of RNA polymerase and DNA gyrase. Synthesis does not occur in extracts made from *polA*$^-$ bacteria, which indicates the participation of DNA polymerase I (107, 117). In vivo ColE1 replication also requires DNA polymerase I (118) and RNA polymerase (119).

The in vitro origin of replication has been located by analysis of the 6S L fragments (120). The precise location where synthesis of the DNA in the 6S L fragments begins was determined by treating the DNA with alkali to remove a possible primer RNA, labeling the 5' ends exposed by the alkali with ^{32}P, and then digesting the DNA with restriction enzymes. The sizes of the labeled fragments indicated the position of sites at which DNA synthesis begins. These sites, which are defined as the origin of ColE1 DNA replication, are located almost exclusively at any of three neighboring nucleotides. The presence of specific RNA-DNA linkages has been confirmed by nearest neighbor analysis of fragments synthesized with [α-^{32}P]dNTP (121). The origin of in vivo replication appears to be the same site used as the origin for in vitro replication (122).

Some DNA is synthesized on supercoiled ColE1 DNA by a mixture of RNA polymerase and DNA polymerase I (123). A protein, later identified as RNase H, has been isolated from cell extracts by virtue of its ability to stimulate DNA synthesis at the specific origin when added to the enzyme mixture. In the presence of RNase H, a majority of the products of synthesis (about 300 nucleotides in length) initiate at the specific origin described above. Without RNase H, only 20% as many fragments are made, none of which begin at the specific origin (123). RNase H appears to process the RNA, thus creating a specific end at which DNA synthesis can begin. This conclusion is based on the observation that the distribution of the 5' ends of the DNA molecules among the several nucleotides at the origin is altered by the exact concentration of RNase H in the reaction mixture (123). The 5' end of the primer for DNA synthesis has not yet been identified.

As described above, the synthesis of ColE1 by a mixture of DNA polymerase I, RNA polymerase, and RNase H occurs in the absence of DNA gyrase even though this enzyme is required for synthesis in extracts. Addition of gyrase to the mixture results in the formation of long L-strand DNA although H-strand DNA is still not made (123). However, the addition of DNA gyrase does not alter the number of molecules on which synthesis initiates as long as a supercoiled template is used. It has been inferred from this that a role of DNA gyrase in synthesis of 6S L fragments by extracts is to counteract the action of topoisomerases that reduce the superhelical density of the template (123).

The region of ColE1 DNA that is essential for its replication has been deduced from the properties of plasmids obtained by ligating various restriction fragments of ColE1-type plasmids to other replicons or to a fragment that confers a selectable property (124–126). The results show that only a small portion of the plasmid genome is required for its replication and maintenance in bacteria. At this time, the essential region is estimated

to contain no more than 580 base pairs of which only 13 base pairs are downstream from the origin of replication (125). The essential region must specify the primer RNA and, in addition, may carry a recognition site for a hypothetical enzyme that ensures separation of daughter molecules (107, 127). The possibility that the region also encodes a protein required for plasmid maintenance, but not for replication per se, has not been excluded. An RNA about 110 nucleotides in length (128) is transcribed from the part of this region which is most distant from the origin of replication. It has been proposed that this RNA is processed and then hybridizes to the origin region where it serves as the primer for DNA synthesis (125). So far, no supporting data are available.

ColE1 DNA replicates unidirectionally, and synthesis of both strands is usually coordinated (113, 129, 130). Once synthesis of the 6S L-DNA starts, RNA polymerase is no longer needed (114). On the other hand, some functions that do not participate in the synthesis of 6S L-DNA are involved in the subsequent DNA synthesis that yields complete molecules. These functions include *dnaB, dnaC, dnaZ,* and DNA polymerase III [(107, 107a, 117, 131, 132); Y. Sakakibara, personal communication; T. Itoh and J. Tomizawa, unpublished results)]. Participation of *dnaG* protein in ColE1 DNA replication in vivo has been suggested (133).

Two possible mechanisms for the synthesis of ColE1 DNA are schematically presented in Figure 1 and can be summarized as follows: (*a*) Synthesis of the L fragment from the origin requires a unique set of proteins. A different set of proteins is required for synthesis of the rest of the molecule; (*b*) The L strand, as a leading strand, is extended continuously (Model *A*) or else discontinuously (Model *B*); (c) The H strand, as a lagging strand, is synthesized discontinuously.

In either model, the use of a unique origin for ColE1 DNA replication results from some site-specific property of the mechanism used to synthesize the first portion of the L strand. Since this synthesis separates the strands of the duplex molecule, synthesis of the first H strand fragment can now

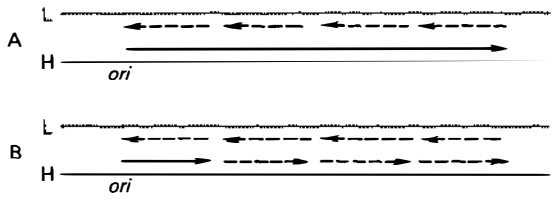

Figure 1 Schematic representation of DNA fragments synthesized on the H and L strands. The continuous and discontinuous arrows represent DNA fragments synthesized by different mechanisms. The two models, *A* and *B*, are described in the text.

initiate on the exposed L strand by use of a second mechanism that requires a single-stranded template and other gene products. It is with respect to the synthesis of the rest of the molecule that the models diverge.

Model *A* supposes continuous synthesis of the L strand. This is suggested by results obtained with purified enzymes. These show that extensive elongation of the L strand can occur when purified RNA polymerase, RNase H, DNA polymerase I, and DNA gyrase are used to synthesize ColE1 DNA (123). If this model is correct, the requirement for certain gene products (e.g. the *dnaB* protein) during extension of the L strand in extracts, may simply result from a necessity to coordinate the synthesis of H and L strands in extracts and in vivo.

Model *B* supposes discontinuous synthesis of the L strand. This possibility has been suggested by experiments that used extracts made from bacteria containing a temperature-sensitive DNA ligase (134). In these extracts, DNA fragments of about 7S are synthesized on various regions of both strands of ColE1 DNA at high temperature. Control experiments indicate that the small fragments are not generated by a repair process that acts on misincorporated nucleotides (135). Thus there is some reason to think that the fragments are formed as intermediates in the elongation of both strands.

Activation of the Origin by Transcription

Transcriptional activation is a concept that arose from studies of the replication of phage λ (136, 137). Although the mechanism that activates λ DNA replication is not fully understood, the concept itself is important in considering the initiation of DNA synthesis on double-stranded DNA.

Replication of λ DNA initiates at a fixed origin and proceeds bidirectionally (138). The characterization of stable plasmids composed entirely of small portions of λ DNA has shown that all the information needed for the replication of λ DNA lies between 78% and 83% on the physical map (139) (Figure 2). A more precise location of the origin itself is given by mutants (now called *ori*$^-$) which have a *cis*-dominant defect in replication and are, therefore, believed to have an altered origin of replication (137, 140). The mutations responsible for the defect map in gene *O* (140) and, as will be discussed below, are small deletions or, in one case, a single base change (141, 142).

In vivo, the replication of λ DNA depends on the phage *O* and *P* proteins (143) and on several bacterial proteins including the *dnaB, dnaG, dnaJ* (144, 145), and *dnaK* (144, 145) products, DNApolymerase III, and RNA polymerase (146, 147). Genetic complementation tests show that the *O*-like proteins of various lambdoid phages cannot substitute for each other, although in some cases, their *P*-like functions can (147–149). Measurements of the ability of hybrids formed by recombination between λ and ϕ80 or

82 to make use of the *O* function of these various phages show that the inability of λ to use other *O*-like proteins is due to sequences present in the N-terminal region of its own gene *O* (150). A similar analysis suggests that the ability of λ to use the *P*-like functions of other phage may be governed by the C-terminal region of the *O* protein (150). The interaction of the *O* and *P* proteins has also been inferred from the ability of some gene *P* mutations to suppress a gene *O ts* mutation (151). Since the *P* protein has been shown to interact with the *dnaB* protein (152, 153), it appears possible that all three proteins may act as a complex.

Even when all necessary gene products are supplied in *trans,* λ DNA cannot replicate in the presence of repressor, an effect which is termed replication inhibition (154). Genetic studies of replication inhibition suggest that it is due to inhibition of rightward transcription from the P_R promoter, and that either the transcript need not be translated, or else a required translation product is *cis*-acting (136).

The possibility that a *cis*-acting protein is required is unlikely because of the properties of some λ mutants (ri^c) that overcome replication inhibition (137). These were obtained by mixed infection of a λ lysogen with λ and a heteroimmune helper phage. After several cycles of selection, two types of mutants able to replicate in the presence of λ repressor were obtained; those in one class express the *O* and *P* functions constitutively, while mutants of the second type (ri^cD, ri^c5b) express the *P* function constitutively as the result of mutations mapping within the *O* gene itself (155). Even in the presence of repressor, the ri^cD mutation allows transcription that starts at the site of the mutation and proceeds to the right (156). Since the ri^cD mutation enables λ to replicate when transcription from other promoters is repressed, any *cis*-acting protein should be encoded by the

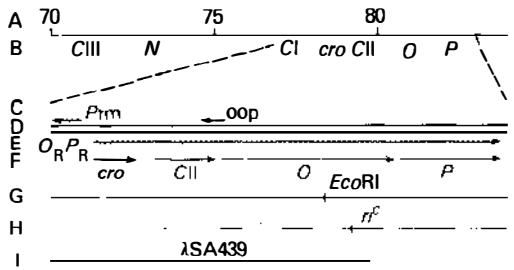

Figure 2 Maps of a portion of phage λ genome that controls transcription and DNA synthesis. The information is taken from references cited in the text. *A,* distance from the left end of the DNA molecule expressed as a percentage of its total length; *B,* approximate position of genes; *C* and *E,* sites of promoters and direction of transcription; *D,* the region contained in λ*dvh*93; *F,* extent and direction of translation; *G, Eco*RI cleavage site; *H,* location of the ri^c mutations; *I,* the region retained in λSA439.

region to the right of the mutation. However, most of this region is deleted in a mutant (λSA439) (Figure 2) which can, nonetheless, synthesize DNA when the *O* and *P* proteins are supplied in *trans* (157). Since the only region present in λSA439 that can be transcribed from ri^cD is an internal portion of the *O* gene, transcription from ri^cD should not be required for synthesis of a *cis*-acting protein (137).

It has been proposed that the transcription could act by altering the structure of the origin region and/or by moving the origin to a site in the cell where it can be replicated (137). These explanations were proposed before the concept of RNA priming of DNA synthesis was established, and the first possibility may now be divided into two different ones, depending upon whether the transcript is to be used as a primer for DNA synthesis or not. While it is not yet possible to completely rule out any of these proposals, recent results obtained through the cloning and sequencing of various regions around the origin of replication have provided some additional insight into the problem.

Digestion of λ DNA by *Eco*RI produces a fragment containing a region that extends from the immunity region to a point inside *O* gene (66%–81% on the physical map). When this fragment is inserted into a λ-φ80 hybrid vector lacking the λ origin region, the composite phage now shows λ-specific replication (158). This result suggests that the region covered by the fragment contains the λ origin of replication. Furthermore, a combination of genetic analysis and DNA sequence determination shows that at least four of the ori^- mutations described above cluster in a small region just to the left of the *Eco*RI site within gene *O* (140, 141, 155) (Figure 2).

In an attempt to delimit the required region to the left of the *Eco*RI site, portions of the λ DNA in the composite phage have been deleted by in vitro techniques (159). The results show that the phage can still grow when all λ DNA to the left of the middle of the *cI* gene has been removed. Furthermore, the additional deletion of nearly all the DNA lying between the P_R promoter and a site well within gene *O* still gives a phage that can replicate.

These results show that, at most, the 190 base-pair segment of DNA just to the left of the *Eco*RI site is required in conjunction with the P_R promoter to give *O* protein-dependent replication. Remarkably, the two ri^c mutations in gene *O* are located to the right of this *Eco*RI site (150). Presence of the ri^c5b mutation causes transcription which begins 135 ± 5 base pairs to the right of the *Eco*RI site and proceeds to the right (150). Thus there is no apparent requirement for transcription in the region believed to contain the origin. This is true even if the region extends as far as 20 base pairs to the right of the *Eco*RI site, as has been reported (142).

An important objection to such a conclusion is raised by the possibility that the ri^c mutation creates a new origin for λ replication. This is excluded

by the following experiment (150). Phage 82 and λ are related phages but their *O* proteins are phage-specific. A hybrid (*rep*82 : λ) was constructed in which the origin region to the left of the *Eco*RI site is derived from 82 and the region to the right from λ. This phage requires the 82-type *O* protein. If the ri^c mutation creates a new origin, *rep*82 : λri^c phage should not require the 82-type *O* protein for replication under the repressed condition. In fact, the 82-type *O* protein, but not the λ-type *O* protein, supports replication of the phage. Therefore, it appears likely that the ri^c mutation does not create a new origin. Rather, these results argue that transcription from the ri^c5b mutation somehow activates the normal origin.

The results described above indicate that a small region to the left of the *Eco*RI site is sufficient for λ-specific initiation of replication when the *O* and *P* proteins are supplied in *trans*. A similar conclusion has been drawn from independent experiments in which composite plasmids were created by insertion of restriction fragments from λ*dv* into a ColE1-type vector, and the maintenance of the plasmids under *polA*$^-$ conditions was determined (142). This time, the *O* and *P* proteins were supplied by a second hybrid plasmid which contains all of λ*dv*. Since the vector itself is not maintained in the absence of *polA* function, a positive result indicated the presence of a functional λ origin.

The results show two regions of importance; one is a 199 base-pair segment (the A region) directly to the left of the *Eco*RI site, the other an 89 base-pair segment (the B region) of the *cII* gene. When a plasmid contains only the B region, it can replicate under *polA*$^-$ conditions, but only if the segment containing the B region is transcribed. Results obtained through use of a somewhat different type of complementing plasmid than that described above suggest that this replication requires the *P* protein and not the *O* protein. On the other hand, when both the A and B regions are present in the same relative orientation found in phage λ, plasmid maintenance is now dependent on both the *O* and *P* proteins as well as transcription (142).

The cause of disparity between the results obtained with phage and plasmid vectors is not known. It could simply be due to different contexts around the cloning sites. More likely, it may arise from differences in the kind and amount of replication required by the two vector systems. In particular, the analysis of the origin with the phage vector has been done in a way that requires production of phage particles in addition to DNA replication. In the plasmid system, where bacterial growth was measured, there was a requirement that both daughter cells receive some plasmid DNA at the time of cell division.

Some characteristics of the sequence of the DNA (about 160 base pairs) directly to the left of the *Eco*RI site may be worthy of mention (142, 159). The region contains a series of tandem repeats of a 19 base-pair sequence

that has internal dyad symmetry. Upon strand separation, the region can be folded into remarkable secondary structures which are assumed to play a role in recognition of the DNA by the proteins required for DNA synthesis (142, 160). One *ori*$^-$ deletion mutation is located in this region. In addition, there is an inverted repeat of about 30 base pairs that includes the *Eco*RI site near its center. Between these structures, a highly A-T rich sequence (about 40 base pairs), with an asymmetric arrangement of pyrimidines and purines, exists. Two *ori*$^-$ deletions occur in this region. The analogous region of ϕ80 has four nearly complete tandem repeats of a 12 base-pair sequence, a somewhat similar middle region, and an almost identical inverted repeat. The tandemly repeated sequence differs significantly from that of λ.

It has been proposed that the required transcription separates the two strands of DNA in the origin region, thereby permitting the strands to fold into the secondary structure described above. DNA synthesis could then begin on the exposed single-stranded DNA (142). If this explanation of transcriptional activation is correct, it is still not clear how the *ri*^{c} mutations activate the origin.

We now have a fairly thorough knowledge of the genetics and chemical structure of the origin of replication of λ. Nonetheless, we know very little about the biochemical mechanism of initiation of λ DNA synthesis. It is vital to determine the actual site at which DNA synthesis initiates and the biochemical mechanisms that carry out this reaction.

Formation of Nascent Fragments

The process of DNA chain elongation by sequential synthesis and joining of small nascent fragments (Okazaki fragments) of DNA is called discontinuous synthesis (161). Taking into consideration the polarity of chain growth catalyzed by known DNA polymerases, the strand that elongates in an overall direction of 3' → 5' (the "lagging" strand) must be synthesized discontinuously. In principle, the other strand (the "leading" strand), which grows in an overall direction of 5' → 3', can elongate continuously. Nonetheless, it could conceivably be synthesized by a discontinuous mechanism.

Originally, the presence of labeled fragments following a short pulse of radioactive precursor was taken to be evidence for the occurrence of discontinuous synthesis. However, fragments can be formed by post-replication degradation of DNA as, for example, is known to occur during removal of misincorporated nucleotides (135). Therefore, the mere existence of fragments does not itself constitute sufficient evidence of discontinuous synthesis. Rather, one has to know whether or not the fragments are actually formed by synthesis.

One approach to the problem is to examine the fragments for special structures at their 5' end which could specify the mechanism of formation.

Thus fragments labeled during brief pulses have been tested for the presence of a covalently bound RNA primer. Various methods have been applied to detect the DNA-bound RNA and, in a number of cases, its presence has been claimed (162). However, this interpretation of the data has been disputed (7). Even the use of alkali as a specific agent for the removal of RNA bound covalently to the 5' end of the DNA has been criticized on the grounds that 5'-OH termini can also be created by such treatment of the fragments formed during elimination of misincorporated nucleotides (163). Because of these complications, only a direct chemical demonstration of RNA-DNA covalent linkage at the 5' ends of DNA fragments can provide conclusive evidence for RNA priming in the discontinuous mechanism. The results discussed in this section demonstrate the formation of RNA-primed DNA fragments during replication of phage T7 DNA in vitro and in vivo. Similar results obtained with phage T4 and *E. coli* are also described.

DNA is synthesized on both native and denatured T7 DNA in vitro by a mixture of T7 DNA polymerase, and the T7 gene 4 protein (164, 165). When single-stranded DNA is used as template, synthesis is dependent on the presence of rATP and rCTP (166). Sequence analysis of small fragments formed during the reaction has shown that the primer(s) used are pppApCpCpA (166, 167) or pppApCpCpC (104). Although the RNA-primed DNA fragments are formed on native T7 DNA, this DNA is not as effective a template as is denatured T7 DNA. On native DNA, leading strand synthesis may initiate at a nick and the RNA-primed fragments then form on the displaced single strand.

Primer RNA can be synthesized on single-stranded DNA by gene 4 protein in the absence of DNA polymerase and dNTPs (104, 166). In the presence of single-stranded DNA, the gene 4 protein also has a nucleoside 5'-triphosphatase activity (168, 169). When double-stranded DNA is used as template, hydrolysis of NTPs is interdependent with DNA synthesis.

The study of in vivo synthesis of T7 DNA has also led to the identification of RNA-primed fragments (162). A small fraction of the fragments retain a triphosphate at their 5' end and thus must have an intact primer. Most of these have the structure pppApC(p-rN)$_2$ which is consistent with either of the sequences established in vitro. Shorter segments of RNA are found on the majority of the fragments. Since these lack the 5' triphosphate, they are presumed to arise through partial degradation of the tetramer. Their base composition is mainly A and C, again in good agreement with the in vitro results.

An attempt to determine the number of sites at which initiation can occur in vivo has also been made (162). The results suggest that even within a short segment of the T7 chromosome (a 350 nucleotide *Hpa* II fragment),

there are several sites at which synthesis of the fragments can initiate. If so, this suggests that initiation does not require a special sequence other than that of the primer itself. It is not known if the primer is made at the site where it is used.

When the products of phage T4 genes 41, 43, 44, 45, and 62 are incubated with single-stranded DNA, rNTP-dependent DNA synthesis is observed, but only if another protein, called protein X, is also present (170–173). Protein X activity is absent from extracts of cells infected with phage mutant in gene 61, but it has not yet been shown that gene 61 actually encodes protein X (172, 173). DNA synthesis can also occur if nicked ColE1 DNA is used as a template. In this case, the gene 41 and X proteins, as well as rNTPs, can all be omitted, but another T4 protein, the gene 32 product, must be added (172). No synthesis is observed when intact duplex DNAs are supplied as template, and it is possible that additional proteins are required for initiation with such templates.

The T4 chain initiation reaction that occurs on single-stranded DNA requires ATP and CTP, while GTP and UTP stimulate the reaction (172, 173). When the gene 41 and X proteins are incubated with ATP, CTP, and fd (+) strand DNA, short oligonucleotides are synthesized in large yield (173). Most of the products contain six to eight nucleotides. When GTP and UTP are also present, they are incorporated into oligonucleotides of similar lengths. At least two kinds of products are made from ATP and CTP, and at least several kinds when the four rNTPs are used. Some of these oligonucleotides can be used as primer for DNA synthesis when the necessary components are supplied (173).

The results of other experiments partially complement these observations by providing evidence of the in vivo formation of RNA-primed DNA fragments (162). More than 40% of the primers appear to be pentanucleotides with a sequence at their 5' end mainly of pApC. A primer with a 5'-nucleoside triphosphate has not been isolated.

The presence of RNA-primed DNA fragments in *E. coli* has also been demonstrated (174, 175). Small fragments were isolated from a strain carrying a *polAex*$^-$ mutation. The 5' ends of the fragments were labeled with ^{32}P and the fragments then digested with pancreatic DNase or with 3' → 5' exonuclease of T4 DNA polymerase. The digestion products included [5'–^{32}P] ribo-deoxyribo-oligonucleotides containing two to six nucleotides (174). In a similarly designed experiment, the fragments labeled with ^{32}P at the 5' ends were hydrolyzed by alkali. Some label was found in 2'(3'),5'-ribonucleoside diphosphates, predominantly pAp and pGp (175).

In these three cases, where the presence of RNA-primed DNA fragments has been clearly shown, it has not been demonstrated that such fragments are synthesized on both strands in the same region of the genome.

INITIATION OF REPLICATION OF THE *E. COLI* CHROMOSOME

The chromosome of *E. coli* is a circular duplex DNA that is folded into a series of approximately one hundred supercoiled loops. Replication initiates within a defined region on the genome and then proceeds bidirectionally by a semi-conservative mechanism. During the elongation process, one or perhaps both strands are synthesized discontinuously. Genetic studies show that the *dnaB, dnaC,* and *dnaG* functions participate in this process together with functions needed for unwinding, polymerization, ligation, and supercoiling of DNA (8, 10). While information about the elongation reaction has been provided by studies that employ cell lysates or toluene-treated cells (3, 4), as yet no information about the initiation reaction is available from such in vitro studies. Thus the following description of events occurring during initiation is based on analyses of replication in vivo. The region of the DNA where these events are thought to occur will be described in the subsequent section.

Function of Required Gene Products

A number of genes whose products participate in replication of the *E. coli* chromosome have been identified by the study of conditionally lethal mutants (10). Whereas many mutants stop DNA synthesis immediately when shifted to a nonpermissive temperature (elongation-defective mutants), others appear to be able to finish rounds of replication in progress at the time of the shift. This second class of mutants, termed initiation-defective mutants, includes both *dnaA* and *dnaC* mutants. As described below, at least one *dnaB* mutant also falls into this class (176). Mutations in several other genes, (*dnaI, J, K,* and *P*) give this phenotype but are not well characterized (9, 144, 145).

The addition of rifampicin and chloramphenicol to inhibit RNA and/or protein synthesis in *E. coli* also inhibits chromosome replication, but not until replication in progress at the time of addition is completed. Thus some new RNA and/or protein must be synthesized to initiate a new round of replication (10, 177). However, the arrest of RNA synthesis is still inhibitory at a time in the cell cycle when arrest of protein synthesis no longer prevents replication (178, 179). Mutants (*sdr*^{c}) capable of continuing several rounds of chromosomal DNA synthesis in the presence of chloramphenicol have now been isolated (180, 181). Each new round of replication requires the *dnaA* and *C* gene products and is inhibited by rifampicin. Because the *sdr*^{c} mutation is recessive, it has been suggested that the chloramphenicol-sensitive step in wild-type bacteria is the synthesis of a protein that counteracts a negative regulatory function. This regulatory function is believed to be defective in the mutants.

Experiments that examine the interaction of various initiation-defective mutants and the two drugs mentioned above suggest a particular order of events at the time of initiation. For example, if temperature-sensitive *dnaA* or *dnaC* mutants are exposed to a nonpermissive temperature for a time sufficient for ongoing rounds of replication to finish, a new round of replication initiates when the cells are returned to the permissive temperature whether or not chloramphenicol is present (182, 183). This suggests that the necessary protein synthesis can occur in the absence of either gene product. On the other hand, when rifampicin is present at the time of return to permissive temperature, the *dnaA* mutant cannot resume replication although the *dnaC* mutant can (183–185). This suggests that the action of the *dnaA* product precedes or is concurrent with the rifampicin-sensitive step, but that in either case, both precede the reaction that requires the *dnaC* product.

One biochemical function of *dnaA* protein has been suggested by the observation that some *dnaA* mutations are suppressed by mutation to rifampicin resistance (186, 187). These suppressing mutations occur in the structural gene for the β subunit of RNA polymerase which suggests, among other possibilities, a direct interaction of the *dnaA* protein and RNA polymerase. Obviously, such a proposal is consistent with a requirement for active *dnaA* protein during the rifampicin-sensitive step of initiation.

A second kind of suppression of *dnaAts* mutations is observed when the DNAs of any of a number of plasmids or phages are present in the bacterial chromosome. For example, integration of the F or R factors into the chromosomes allows the growth of *dnaAts* bacteria at nonpermissive temperatures (188–191). The integration of certain phages, such as P1 (192) and P2*sig5* (98), can give a similar result. When the F factor (M. G. Chandler and L. Caro, personal communication), R100-1 factor (193), or the phage P2*sig5* (194, 195) is used for this integrative suppression, the origin of chromosome replication in cells growing at high temperature is in the region where the integrated DNA resides. However, when such cells are grown at low temperature, replication of the chromosome starts frequently at the *E. coli* origin (196). The simplest interpretation of these experiments is that the *dnaA* protein is required for initiation of replication of the *E. coli* chromosome only at its own origin.

For some time, all *dnaB* mutants have been classified as elongation-defectives and the possibility of a role for the *dnaB* protein in initiation was speculative. Now, the characterization of *dnaB252,* an exceptional mutant that has an initiation-defective phenotype (176), provides evidence for direct participation of the protein in initiation. This temperature-sensitive mutant cannot begin, but can continue chromosome replication at high temperature. Furthermore, unlike other *dnaB* mutants, it can support replication of phage λ at high temperature (197). The purified *dnaB252* protein

is unable to aid in the synthesis in vitro of ϕX (–) strands, but does retain a DNA-dependent ATPase activity (197) characteristic of the wild-type *dnaB* protein (198, 199). In contrast to this result, the *dnaB* protein of an elongation-defective *dnaBts* mutant has been found to have a temperature-sensitive ATPase activity (199). These observations suggest that the requirement for *dnaB* protein during both elongation of the *E. coli* chromosome and replication of λ DNA can be satisfied by the ATP-consuming activity of the protein, while the initiation of chromosome replication and the synthesis of ϕX DNA may require another activity, or possibly both. Such a difference in the mechanisms of action of the *dnaB* protein during initiation and elongation of the chromosome may explain the properties of another *dnaBts* mutant, BT165/70. A shift to high temperature reversibly inactivates the ability of this mutant to synthesize DNA, but irreversibly damages its ability to initiate new rounds of synthesis (200).

As described above, results obtained with the study of ϕX DNA replication in vitro show that the *dnaB* and *C* products act in concert. However, in contrast to the initiation-defective class of *dnaC* mutants, the mutant *dnaB252* cannot initiate new rounds of replication when returned to low temperature in the presence of rifampicin. If the *dnaB* and *C* proteins also act together during initiation of chromosome replication, the *dnaB252* mutant must somehow be less efficient in making use of the *dnaA*-dependent transcript than are the *dnaC* mutants so far tested. This might occur if the *dnaB252* product is relatively slow to renature and the transcript is unstable.

In concluding this section, we would like to present three models for how initiation of replication of the *E. coli* chromosome occurs. Because of the lack of sufficient information about the biochemical process that initiates replication of *E. coli* chromosome, the models are based on knowledge obtained with small genomes as described in previous sections, and on fragmentary information about chromosome replication obtained with *E. coli* in vivo. Regulatory mechanisms have not been taken into consideration.

1. RNA polymerase "activates" the origin, but the *dnaG* protein primes initiation of chromosome replication. In this model, RNA polymerase somehow activates the origin, but does not synthesize the primer. For example, transcription could separate DNA strands in the region of the origin and thus provide a single-stranded DNA template for primer synthesis performed by the *dnaG* protein. To ensure that this only occurs at the origin, one assumes that a special structure in the origin region stabilizes the separated strands, and/or that the region contains a specific attachment site either for the *dnaG* protein or for other proteins that mediate its binding to the DNA.

2. RNA polymerase primes initiation of chromosome replication. In this model, the first primer for DNA synthesis is made by RNA polymerase. Thus DNA synthesis could stabilize the separated strands and allow transcription by the *dnaG* protein to initiate lagging strand synthesis. In either this or the previous model, the lagging strand may extend through the region where the leading strand initiates, thus becoming the leading strand for replication of the chromosome in the opposite direction. In this way, initiation of bidirectional replication could be accomplished.

3. A nick at the origin triggers initiation of chromosome replication. Following the nick at a specific site, the 3' end at the break would serve as primer. Once synthesis begins, the 5' end would be transferred to reform an intact parental strand. Replication in the other direction could be initiated by the *dnaG* protein as described above. Transcription by RNA polymerase would somehow be required in a preparatory stage before the nicking occurs.

So far, no evidence points to one of these as the mechanism operating at the origin. Obviously, identification of the nature of the primer for initial synthesis at the origin is crucial. An RNA species that seems to be synthesized under *dnaC*$^-$, but not *dnaA*$^-$, conditions has been described (201). The RNA has been termed "origin-RNA" though neither synthesis nor utilization of the RNA in the origin region has been demonstrated. There is no evidence that this RNA has a role in initiation of replication from the normal origin.

The third model appears the least satisfactory because it provides no clear role for RNA polymerase. Nonetheless, there are two known situations where initiation probably occurs in the manner described by this model. One occurs when phage P2*sig5* is integrated in a *dnaAts* strain, thus suppressing the temperature-sensitive phenotype. For replication to occur, the phage gene *A* protein, a specific endonuclease (202), has to be active (98). Following initiation, replication appears to proceed unidirectionally from the site of phage integration and only later to go in both directions (194, 195). The other situation occurs during bacterial mating. The replication that occurs during transfer of the chromosome is probably carried out by the third mechanism except that here the 5' end at the nick is transferred to the recipient bacteria (203, 204).

Detection of the Origin of Replication

Genetic studies have located the origin of replication within the vicinity of the *ilv* gene (205, 206). A physical map of DNA in the origin region has been obtained by labeling the chromosome of *dnaA* and *dnaC* temperature-sensitive initiation mutants just at the start of a synchronous round of replication (207). The distribution of radioactivity among restriction frag-

ments of the pulse-labeled DNA showed that the origin of replication was located within or very near a *Hin*dIII segment of 1.3 kb (kilobase) in length. This segment is itself situated within an 8.6 kb *Eco*RI segment. Studies of various plasmids that carry the *ilv* region have now allowed the refinement and correlation of the genetic and physical maps. This line of work began with the isolation of an F' factor carrying the putative origin of replication.

While most F' factors are maintained in F^- bacteria, only a few spontaneously arising F' factors (F' *poh*$^+$) are readily carried by *Hfr* bacteria (208). The *poh*$^+$ phenotype is invariably associated with the presence in the F' factor of the *bglB-rbsK* region (209) (Figure 3). These observations are thought to indicate that the site where *E. coli* chromosome replication initiates lies somewhere within the region. The presence of the bacterial origin on F' factors that contain the *bglB-rbsK* region has also been used to explain the finding that strains that carry the factors exhibit slow growth and disturbed cell division (210).

The physical and genetic maps were correlated by restriction enzyme analysis of the DNAs of various F' factors containing portions of the *bglB-rbsK* region (211) (Fig. 3). This correlation was also established by experiments in which restriction fragments formed by *Eco*RI digestion of the entire *E. coli* genome were ligated to a DNA fragment containing a gene responsible for ampicillin resistance. By using this DNA to transform bacteria to ampicillin resistance, three self-replicating plasmids were isolated (212, 213). These were all found to contain the 8.6 kb *Eco*RI segment. One of the plasmids (pSY211) can be integrated into the *E. coli* chromosome at sites between the *uncA* and *rbsK* genes by *recA*-mediated recombination, which indicates that the plasmid contains *E. coli* DNA coming from the region close to these genes (Figure 3).

In an independent set of experiments, a series of λ-transducing phages containing the region around the *asn* gene were isolated (214, 215). The *asn* gene has been shown to lie in the 8.6 kb *Eco*RI segment (211). Some

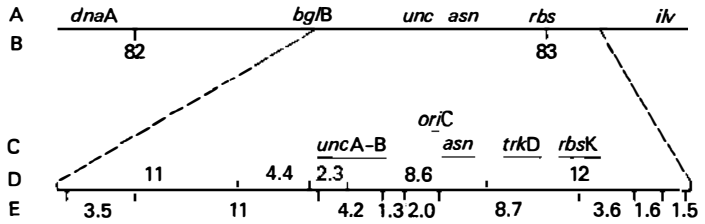

Figure 3 Genetic and physical maps of the region around the *E. coli* origin of replication. *A* and *C*, approximate position of genes; *B*, map positions in terms of minutes; *D* and *E*, sites of cleavage by *Eco*RI (*D*) and *Hin*dIII (*E*), and the sizes of segments in kilobases.

of the λ*asn*-transducing phages were found to establish themselves as plasmids following infection of a λ lysogen. Genetic and physical analyses of various deletion derivatives obtained from the phages led to the conclusion that the origin of replication used by these λ*asn* plasmids is located between the *unc* and *asn* genes (214, 215).

The functional origin present in the λ*asn* plasmids and in pSY211 has been termed *ori*C, even though this name was originally proposed as a designation for the origin of replication of the bacterial chromosome (208). To avoid confusion, we also use *ori*C to indicate the origin of replication of these plasmids, but will return below to the question of the relationship of *ori*C and the chromosome origin.

Using DNAs of deletion mutants of plasmids that have the *ori*C of pSY211 or of λ*asn,* the nucleotide sequence of the region containing *ori*C (about 500 base pairs) has been determined (213, 216–218). The sequence shows a high degree of repetitiveness and a remarkable degree of homology with the sequence of the putative replication origin of phage λ and with the sequence of a stretch of the G4 genome upstream from the site where the primer for synthesis of the (–) strand initiates. This homology is represented twice in the *ori*C region, once in each orientation with respect to the rest of the chromosome. By analogy to the G4 initiation mechanism, the homologous sequence is presumed to be a recognition site for the *dna*G protein (213, 217). However, much of the sequence that is common to the *oriC,* λ, and G4 DNAs is not found in the corresponding region of the ST-1, ϕK, and α3 genomes (51).

In concluding this section, we would like to examine the criteria that establish *ori*C as the "real" *E. coli* origin of replication. First, only one to three copies of the plasmid carrying an intact 8.6 kb *Eco*RI segment are present in the cell, and the plasmid is readily lost (216, 219). Cells carrying the plasmid multiply slowly and it appears possible that the *ori*C of the plasmid and the origin of the chromosome compete for a limiting substance in the cell. However, deletion of a portion of the plasmid DNA allows more rapid multiplication by its host and increases the number of plasmid copies per cell. The portion of the plasmid that is lost in such deletion derivatives includes part of the *unc* genes whose products participate in electron transport (219). Further analysis is required to establish the cause of this effect and, in particular, to determine whether or not overproduction of the *unc* gene products causes poor growth.

Replication of an *unc* deletion derivative has been shown to require the *dnaA* and *dnaC* proteins, and to be quickly inhibited by the addition of rifampicin (219). However, the addition of chloramphenicol gives only gradual inhibition (219). Thus, at least in part, replication of the plasmid resembles that of the *E. coli* chromosome. Unlike the λ*asn,* plasmid

pSY211 and the two other plasmids obtained simultaneously were selected without the use of any chromosomal markers. Nonetheless, all these isolates contain the same 8.6 kb *Eco*RI segment. As described above, independent experiments indicate that this segment is the first region of the chromosome to replicate following the shift of a *dnaA* or *dnaC* mutant to a permissive temperature (207). These results suggest that the *oriC* may represent a unique origin for chromosome replication and that no other region of the DNA may be able to endow the ability to self-replicate.

In the assessment of this information, however, two observations appear important. First, sequences that can serve as functional initiation sites, but normally do not, may be present in a chromosome. As discussed above, the analysis of plasmids containing the phage λ origin (142) provides an example of this and, in addition, points to the possibility that such sequences may come from a region close to the "real" initiation site itself. This means that chromosomal location, while useful, is not a sufficient criterion for the identification of the normal origin.

Second, the details of the strong selection used to isolate plasmids like pSY211 may influence the uniqueness of the products that are obtained. In fact, when the procedure used to isolate pSY211 was repeated, this time using an F^- rather than Hfr strain as host, a self-replicating plasmid that does not contain the 8.6 kb *Eco*RI segment was isolated (220). The origin present on this plasmid is not homologous to *oriC,* and so has been named *oriJ.* Neither the location of *oriJ* on the *E. coli* chromosome nor its role, if any, in the normal mechanism of initiation of chromosome replication has been established.

REGULATION OF DNA REPLICATION

A major question in understanding the regulation of DNA replication is how the number of copies of a genetic element is maintained in the cell at a certain characteristic value, the copy number. It is likely that this maintenance is achieved through regulation of the frequency and timing of initiation of replication with respect to growth of the cell. To illustrate the problems involved, we briefly discuss several proposed mechanisms of regulation. No attempt is made to cover the literature.

The replicon model (221) was an early attempt to provide a framework for understanding regulation and, in particular, to show that the same kinds of elements that regulated gene expression could serve to regulate replication. The model states that (*a*) a genetic element such as a chromosome or episome constitutes a unit of replication, or *replicon,* and that (*b*) the replicon itself carries two specific determinants. One determinant is a structural gene that controls the synthesis of a specific *initiator,* while the other,

the *replicator,* is a specific recognition element (analogous to the operator) upon which the initiator acts to permit replication. Although presented as a positive regulatory mechanism (221), the model itself does not specify how the amount of active initiator in the cell is to be regulated. Therefore, in its basic structure, the model does not predict the actual mode of regulation.

In their discussion of the replicon model, its authors provided several examples to illustrate how the level of active initiator might be determined by negative control of initiator synthesis or by positive control of the activity of the initiator once made. As an example of the former, they proposed that the phage repressor blocks synthesis of an initiator in λ lysogens. As an example of the latter, it was proposed that the bacterial chromosome is attached through its initiator to the bacterial membrane, and that initiation is positively regulated by growth of the membrane (222). Though meant as an example, the latter proposal has been frequently included as part of the replicon model itself.

Recently it has been suggested that the phenomenon of autoregulation (223) provides an attractive mechanism for the regulation of initiator production (224). The replication of phage λ as a plasmid provides an instructive example. Under certain conditions, λ uses its own specific replication functions to replicate as a plasmid (225, 226). Study of deletion variants called λ*dv* (225) has shown that three phage products are invariably required for plasmid formation (227, 228). These are the *cro, O* and *P* proteins which are produced as a result of transcription from promoter P_R (Figure 2). The *O* and *P* proteins act as initiator, while the *cro* protein represses transcription and thereby regulates the supply of all three proteins (229). Through this simple feedback mechanism, growth of the cell can lower the intracellular concentration of the *cro* protein, thus triggering transcription and replication.

So far, all bacterial genetic elements examined have one or possibly a few specific sites where replication initiates. Thus the existence of replicators appears to be universal. The occurrence of initiator-like proteins is widespread, but there is, as yet, little direct evidence that these proteins actually regulate replication. Furthermore, it is now clear that an initiator is not produced by all genomes. An example is provided by the plasmid ColE1 whose replication does not require any plasmid-encoded proteins. In this case, replication could be limited simply by the synthetic capacity of the host cell, or possibly by a negatively acting plasmid function.

A third type of model proposes that a negatively acting element, rather than an initiator, regulates the rate of initiation (230, 231). In this model, regulation is accomplished by coupling synthesis of the negative element, or inhibitor, to replication of the gene that produces it. Thus, each initiation event leads to replication of the gene and production of the inhibitor which

then represses further initiation. Growth of the cell reduces the concentration of the inhibitor and thereby increases the probability of initiation. Formally, this model can explain both the maintenance of replicons at a unique copy number and the timing of initiation with respect to the cell cycle. As does the autoregulatory model described above, it employs a feedback mechanism that links gene expression and replication.

This model has been used to explain the replication properties of several plasmids, one of which is the sex factor F. In exponentially growing Hfr cells, where F is found as part of the chromosome, replication usually initiates at the chromosomal origin and not at that of the integrated F factor (232). To explain this by means of the negative regulation model, it is necessary that the effective copy number of F in the Hfr cells be higher than it would be in F^+ cells. This would happen if, on the average, the F DNA replicates sooner in the cell cycle when integrated than it does when present as a plasmid. Thus repression can be maintained in the absence of initiation at the F origin. There is now some experimental support for such an increase in the effective copy number of F in Hfr strains (233, 234).

Studies of the composite plasmid pSC134 have yielded results similar to those obtained with the F factor, and have been used to support the existence of replicon-specific negative regulation of initiation (235). This plasmid contains the entire genomes of two compatible plasmids, pSC101 and ColE1. In wild-type bacteria, the composite plasmid uses its ColE1 replication origin exclusively, and exists at a copy number of 15 per cell, or about three times higher than that of pSC101 in the same strain. In their discussion of this result, the authors argue that the turn-off of the pSC101 origin can be most easily explained by the existence of a specific inhibitor of the pSC101 origin. According to the model presented above, the frequent replication of pSC101 DNA present in the composite plasmid should lead to overproduction of a specific repressor of the pSC101 origin (235).

A regulatory model that makes use of an initiator can also explain the results obtained with pSC134. This model requires that (*a*) the rate of initiator production be independent of the number of copies of the replicon present, (*b*) all or nearly all initiators be bound to the DNA at any time, and (*c*) multiple initiator molecules be bound to the same replicator for initiation to occur. If binding of the initiators is random, then an increase in the number of copies of the replicon will decrease the chance that any replicator has the required number of bound initiators, and fewer initiation events will occur. The required control of synthesis of the initiator could be readily obtained with an autoregulatory mechanism of the type described above. As has been observed elsewhere, a similar effect might be observed if a host function limits initiation at the pSC101 origin (231).

This model can also describe much of what is known about the control of F factor replication. However, by introducing it here, we do not mean to imply that it provides a better explanation of the pSC134 or F factor data. Rather, our intention is only to point out that the response of an initiator-type mechanism to a change in DNA concentration can be dictated by the exact details of initiator supply and action.

To date, the only genome whose regulatory mechanism is clearly established is λ*dv*. While each of the models described above provides some insight into how the initiation of replication can be regulated, much remains to be learned.

CONCLUSIONS

Because primers for DNA synthesis play a central role in the initiation of DNA replication, much of the work reviewed here has concerned the reactions involved in primer formation. The known initiation mechanisms require synthesis of an RNA primer, or else the nicking of one strand of a duplex DNA template. Synthesis of the RNA primers can occur on single- or double-stranded templates, and, in at least one case, post-transcriptional modification plays an important role in forming the primer. In some cases, the primer is made at a unique site on the template, whereas in others, a primer may be produced at a large number of sites.

Knowledge of these mechanisms has come primarily from the study of in vitro DNA synthesis on small single- or double-stranded DNAs. The initiation mechanisms employed by large genomes are poorly understood, in part because of the difficulty of in vitro experimentation with large DNA molecules. The use of modern genetic techniques to construct small plasmids that contain portions of the large genomes holds promise of rapid progress in the understanding of how such genomes initiate and regulate their replication.

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