Leading Strand Synthesis of R1 Plasmid Replication in Vitro Is Primed by Primase Alone at a Specific Site Downstream of oriR*

(Received for publication, November 30, 1988)

Hisao Masaï and Ken-ichi Arai
From the Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304

By using an in vitro system for R1 plasmid replication dependent on a plasmid-encoded repA protein and host dnaA protein, 5' ends of the nascent leading strand were located at positions 1986–1992, some 380 base pair downstream of oriR. Analyses of early replication intermediates generated in vitro in the presence of dideoxy TTP also indicated that replication initiates about 400 base pair downstream of oriR and proceeds unidirectionally. When a 418-base single-stranded DNA from position 1778 to 2195, derived from the leading strand template, was cloned onto an M13 vector, the chimeric single-stranded phage could be replicated in vitro with only single-stranded DNA binding protein, primase (dnaG gene product), and DNA polymerase III holoenzyme. Furthermore, the priming occurred at a site identical to leading strand initiation. These results strongly suggest that the leading strand synthesis is primed by primase alone. The lagging strand synthesis is specifically terminated at position 1515 or 1516 within oriR, preventing further leftward fork movement. Based on these results, a scheme of R1 plasmid replication is presented.

Initiation of DNA replication of bacterial replicons requires a defined DNA segment, which is usually called the "replication origin" (1–3). Although these replication origin sequences are sufficient to support DNA replication of a given replicon both in vivo and in vitro, actual sites of initiation of leading or lagging strand synthesis do not necessarily coincide with these "origin" sequences. In some cases, the origin sequence is the site for assembly of a mobile replication machinery, which initiates chain elongation outside of the origin. In complementary strand synthesis (SS to RF)1 of φX174 phage DNA, the initial prepriming complex, which is assembled at a specific location (protein n' recognition site) on the genome, moves processively on the single-stranded DNA in a direction opposite to chain elongation and synthesizes RNA primers at multiple sites (4, 5). On the other hand, primer RNAs are synthesized at a fixed position within the origin sequences in replication of G4 or M13 phage DNA (6, 7). Mapping of RNA-DNA junctions in replication of the duplex oriC plasmid containing the replication origin of Escherichia coli chromosome revealed that they are scattered in the left half within oriC on the counterclockwise strand and to the left of the oriC sequences on the clockwise strand (8). Similarly, RNA-DNA transition sites were identified on both sides of replication origin of λ phage DNA (9, 10). For these replicons, it has been demonstrated that primers are synthesized at multiple sites in the vicinity of the replication origin and that their locations are distributed asymmetrically between both strands. Among replicons known to replicate through Cairns-type mechanism (which is through θ-type intermediates and not by rolling circle or protein priming mode), ColEl-type plasmids are the only example, where leading strand synthesis is initiated at a specific site (11). It has also been suggested that lagging strand synthesis of ColEl-type plasmids is initiated by the primerosome assembled at a specific focus on the lagging strand (n' site) (12).

R1 plasmid replicates through Cairns-type intermediates and replication proceeds unidirectionally (13, 14). The replication origin of R1 plasmid has been localized to a 188-bp sequence, which was designated oriR (3). In vitro replication of R1 plasmid depends on the plasmid-encoded repA protein and host dnaA protein, both of which bind to sequences within oriR (15). Host dnaB, dnaC, dnaG, and ssb functions are also required for the plasmid replication in vitro (15, 16). Binding of repA and dnaA proteins to oriR generates a conformational change of the oriR DNA, as revealed by induced sensitivity of the template DNA to nucleases.2 However, mechanism of priming of leading or lagging strand synthesis is unknown.

In this report, we have determined the 5' end of the nascent leading strand synthesized in vitro. Furthermore, primase alone can prime DNA synthesis at the same site on a chimeric single-stranded phage DNA containing the fragment surrounding the initiation site, strongly suggesting primase-dependent initiation of R1 plasmid leading strand synthesis. We have also shown that R1 plasmid lagging strand synthesis is terminated at a specific site within oriR. On the basis of these data, a scheme for initiation, elongation, and termination of R1 plasmid replication is presented.

MATERIALS AND METHODS

Reagents—Sources were as follows: ribonucleotide triphosphates, deoxyribonucleotide triphosphates, and dideoxythymidine triphosphates (dTTP), Pharmacia LKB Biotechnology Inc.; [α-32P]TTP, Amersham Corp.; potassium glutamate and rifampicin, Sigma. E. coli Strains, Plasmid DNA, and Proteins—The strains used are WM434 (dnaA205) (17), FA22 (dnaB1) (18), and PC2 (dnaC2) (19). pMOB45 (10.5 kb) is a derivative of the R1 plasmid runaway replication mutant (20). pHM6045 and pHM6050 were constructed by joining a 1.55-kb fragment from pRP845 (positions 78–1623) or a 3.7-kb fragment from pREP903 (Ref. 3, positions 1 to ~3700) to a 1.1-kb

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 415-496-1260.

1 The abbreviations used are: SS, single-stranded circular DNA; RF, replicative form; ddTTP, dideoxythymidine triphosphate; pol III, DNA polymerase III; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSB, E. coli single-stranded DNA binding protein; kb, kilobase pair(s); bp, base pair(s).

2 H. Masaï and K. Arai, unpublished data.
fragment containing only the β-lactamase gene. RepA protein was purified to homogeneity from an overproducing strain (Ref. 15 and Footnote 2). dnaB and dnaC proteins were purified as described (21, 22). dnaA protein, primase, and DNA polymerase I11 (pol I11) holoenzyme were gifts from A. Kornberg’s laboratory (Stanford University). SSB was purchased from United States Biochemical Corporation (Cleveland, Ohio). Restriction endonucleases and S1 nuclease were obtained from New England BioLabs and Bethesda Research Laboratories, respectively.

In Vitro Replication of Plasmid and Phage DNA—Conditions for in vitro replication of R1 plasmid were as described (15) except that 40 mM potassium glutamate was used instead of potassium chloride. Fraction I was generally prepared from WM434 and was supplemented with 0.1 pg of single-stranded DNA as template and that 20 μg/ml of rifampicin was also included. SS to RF replication with purified proteins was conducted in 25 μl of assay mixtures containing Hapes-KOH (pH 7.6), 40 mM potassium glutamate, 40 mM magnesium acetate, 10 mM; glycerol, 16%; dithiothreitol, 4 mM; bovine serum albumin, 80 μg/ml; ATP, 1.6 mM; CTP, GTP, and UTP, 0.4 mM each; dATP, dCTP, dGTP, and TTP, 80 μM each with [α-32P]TTP at 200 cpm/pmol of total deoxyribonucleotide, rifampicin, 10 μg/ml, single-stranded DNA template, 100 ng; single-stranded DNA binding protein (SSB), 0.8 μg; primase, 20 ng; and pol III holoenzyme, 70 ng. (The pol III fraction used was deficient in β subunit; 75 ng of purified β was also added.) Incubation was at 30 °C for 10 min.

Isolation of Replication Intermediates Synthesized in vitro—In vitro replication reactions of R1 plasmid were conducted in the absence or presence of various concentrations of ddTTP. After 30 min at 30 °C, 200 μl of 10 mM EDTA was added to the reaction mixture, and 20 μl was used to measure acid-insoluble radioactivity. The remainder was treated with phenol, nucleic acid was precipitated with ethanol and was resuspended in 30 μl of TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA) buffer. The ssDNA was digested with restriction enzyme at 37 °C for 1 h in 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 10 mM potassium acetate, 0.5 mM dithiothreitol, and 100 μg/ml of bovine serum albumin. S1 digestion was at 37 °C for 20 min with 0.01 unit of S1 nuclease in 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnSO4, and 5% glycerol. Alkaline digestion of RNA primer was conducted at 37 °C for 20 min in 1.0 M NaOH solution.

Analysis of Products in Electrophoresis—Neat agarose gels were run in TAE (40 mM Tris acetate [pH 7.8], 1 mM EDTA) buffer at 60 V, 50 mA for 1.5 h. SBSR gels were run in TAE (40 mM Tris acetate [pH 7.8], 1 mM EDTA) buffer at 60 V, 50 mA for 1.5 h. After the run, the gel was stained in water containing 0.5 μg/ml of ethidium bromide, a photograph was taken, and the gel was dried and autoradiographed. Alkaline agarose gels were run in TAE (40 mM Tris acetate [pH 7.8], 1 mM EDTA) buffer at 60 V, 50 mA for 1.5 h. After the run, the gel was soaked in 0.1 M Tris-Cl (pH 7.5) for 1 h, dried, and autoradiographed. Neutral polyacrylamide gels were run in TBE (89 mM Tris borate [pH 8.3], 1.2 mM EDTA) buffer. Denaturing polyacrylamide gels contained 6 M urea in TBE buffer. The samples were mixed with an equal volume of 100 mM NaOH, 2 mM EDTA, and 10% glycerol before loading on a gel. The run was performed at 60 V, 50 mA for 1.5 h. The gel was soaked in 0.1 M Tris-Cl (pH 7.5) for 1 h, dried, and autoradiographed. Neutral polyacrylamide gels were run in TBE (89 mM Tris borate [pH 7.8], 89 mM boric acid, 2 mM EDTA) buffer. Denaturing polyacrylamide gels contained 6 M urea in TBE buffer. The samples were mixed with an equal volume of formamide dye (80% (v/v) formamide, 50 mM Tris borate [pH 8.3], 1 mM EDTA, 1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue), placed in boiling water for 2 min, cooled on ice, and applied on the gel. Radioactive size markers were generated by 3'-end labeling of HindIII-digested λ DNA with the Klenow fragment of DNA polymerase I or 5' end labeling of HaeIII-digested 4X174 RF DNA or Maxam-Gilbert reactions of an end-labeled fragment (23).

Construction of Single-stranded Chimeric Phages Containing DNA Fragments Derived from the Basic Replicon of R1 Plasmid—M13-R101 was constructed by inserting a 419-nucleotide-long fragment (from position 1778 to 2195 (24), HinfI fragment filled in with the Klenow fragment) derived from the template strand for the nascent leading strand at the Smal site of M13mp11. M13-R102 and M13-R105 contain a fragment from position 2008 to 2807 (Ndel to Ndel) in the M13mp13 vector. M13-R104 and M13-R105 carry a fragment from position 1921 to 2058 (AuaII to Ndel) in the same vector. M13-R102 and M13-R105 carry the template strand for the nascent leading strand, whereas M13-R103 and M13-R104 carry the template strand for the lagging strand.

RESULTS

Early Replication Intermediates Synthesized in vitro in the Presence of ddTTP—Elongation of DNA chain is terminated, when ddTTP is incorporated instead of TTP (25). Therefore, early replication intermediates can be accumulated by including ddTTP in in vitro reaction mixtures. Fig. 1 shows an analysis of products synthesized in vitro in the presence of various concentrations of ddTTP on a neutral agarose gel or on an alkaline agarose gel. Form I and form II DNA as well as high molecular weight DNA were synthesized in vitro in the absence of ddTTP. With increasing amount of ddTTP, these products disappeared, and new species migrating slightly above form I started to accumulate. On an alkaline gel, nascent fragments of less than full-length size were accumulated in vitro in the presence of ddTTP over 28 μM. These results show that replication intermediates are accumulated in vitro in the presence of ddTTP.

Mapping of Initiation of R1 Plasmid Replication by Restriction Analysis of Early Replication Intermediates—In order to determine where replication of R1 plasmid is initiated, early replication intermediates generated by ddTTP were analyzed by restriction enzyme digestion. pHM6050, composed of a 3.7-kb R1 plasmid fragment containing repA, CIS (26), oriR, and 2.1 kb further to the right (as drawn in Fig. 2) was replicated in vitro in the presence of increasing amounts of ddTTP. The products were digested by RsaI, which generated 11 fragments, and were analyzed on a neutral polyacrylamide gel (Fig. 2). After normalization for the size of the fragments, the relative extent of replication of each fragment was compared. The most intense replication was detected on the fragment from position 1016 to 2334 (fragment 6; the BglII site, located within copB, is taken as position 1 (24), and oriR is from position 1423 to 1611) under the conditions examined.

![Fig. 1. Agarose gel electrophoresis analyses of early replication intermediates synthesized in vitro in the presence of ddTTP. Replication products, synthesized in the standard in vitro replication reaction mixtures with 0.5 μg of pM60B45 DNA in the presence of various concentrations of ddTTP, were isolated and analyzed on a 0.6% neutral agarose gel (A) or on a 1.0% alkaline agarose gel (B) as described under "Materials and Methods."](image-url)
FIG. 2. ddTTP mapping of initiation of R1 plasmid replication. A, replication products, synthesized in vitro with 0.5 μg of pHM6050 DNA in the presence of various amounts of ddTTP, were isolated, digested with RsaI, and applied on a 6% neutral polyacrylamide gel as described under “Materials and Methods.” Fragments generated are numbered in order of size. Size markers are φX174 phage DNA digested with HaeIII. Lanes 8–11 are shown after four times longer exposure than other lanes for better visualization of bands of weak intensities. B, intensity of each band on the above autoradiogram was scanned by a laser densitometer (ULTROSCAN XL, Pharmacia LKB Biotechnology Inc.), and the peak magnitude for each band was normalized by dividing it by the molecular size of each fragment. The data were quantitated within the linear response time of film exposure. The ratio of normalized peak magnitude for each fragment to that of fragment 6 was calculated and presented as relative replication. The results are presented only for lane 7 (48 μM ddTTP), lane 8 (64 μM ddTTP), lane 9 (80 μM ddTTP), and lane 10 (160 μM ddTTP). The RsaI restriction endonuclease map of pHM6050 is drawn below the histogram. The solid line and dashed line in the map represent 3.7-kb R1 plasmid-derived DNA and 1.1-kb pBR322-derived DNA containing β-lactamase, respectively. The wavy arrows indicate locations of initiation sites of the leading strand and termination sites of the lagging strand. The 5’ end of the lagging strand is indicated as a dotted wavy line, because it was not determined as a specific position. A distance from the position 1 (BglII site in capB, Ref. 24) is also indicated below the map.
As the concentration of ddTTP decreased, increased incorporation into fragments located to the right of fragment 6 was detected. In contrast, little increase in incorporation into the fragment to the left of fragment 6 was observed except for fragment 9 which is located next to fragment 6. Incorporation to fragment 9 increased as dTTP concentration decreased, although it reached only half the level of replication of fragment 6 in the presence of 48 μM dTTP. The above results indicate that replication of R1 plasmid initiates somewhere about 400 bp to the right of oriR and proceeds unidirectionally.

5' End of the Putative First Leading Strand—Analyses by restriction digestion described above suggest that R1 plasmid replication is initiated at a specific position on the basic replicon of R1 plasmid. In order to locate precisely the position of the initiation site, products synthesized in vitro were digested by restriction enzymes which cut the template DNA to the right of oriR, and the single strands were analyzed by denaturing polyacrylamide gel. When they were digested by Ndel or EcoRV, fragments consisting of 6–7 base ladders centered around 70 or 128 nucleotides long, respectively, were detected (Fig. 3A). Since Ndel and EcoRV cut pHM6050 at positions 2057 and 2115, respectively, the above results predicted the presence of either a specific 5' end of a nascent leading strand or 3' end of a nascent lagging strand at positions 1985–1991.

In order to determine the strandedness of this nascent fragment, chimeric single-stranded phage DNAs containing each strand derived downstream from oriR were immobilized onto a nitrocellulose filter and were hybridized with the 32P-labeled 130-nucleotide-long fragments generated by EcoRV digestion. The fragments hybridized only with the phage DNA containing the template strand for the leading strand (Fig. 4), indicating that they were derived from the nascent leading strand. Furthermore, alkaline treatment of the products resulted in decrease of the sizes of the fragments by one to two nucleotides (Fig. 3B), suggesting the presence of RNA primer attached to its 5' end. This would also indicate that these nascent DNA fragments with 5' ends around position 1986 are generated by de novo synthesis and not by processing of longer fragments.

These fragments were synthesized in a very early stage of R1 plasmid replication, since they were observed even in the presence of 160 μM ddTTP where very few products longer than 300 nucleotides were synthesized (Fig. 3, C and D). Based on these results, we have concluded that leading strand synthesis is initiated around position 1986. Initiation at position 1986 is consistent with the above restriction analyses of early replication intermediates, which show that the region immediately downstream from this position (positions 2016–2334) is replicated in the earliest stage.

| Leading Strand Template DNA Cloned onto a Single-stranded M13 Vector—Initiation of leading strand synthesis at a specific site suggests the presence of a signal for RNA priming at or near the initiation site. A fragment (positions 1778–2195) derived from the vicinity of the initiation site was cloned into an M13 vector, M13mp11. Single-stranded DNA from the chimeric phage M13-R101 was isolated, and replication activity for SS to RF conversion was examined in vitro. A partially fractionated enzyme system, Fraction II, prepared from WM434 (dnaA205) could support efficient formation of RFII and RFII from the single-stranded DNA template either in the presence or absence of dnaA protein (data not shown). In order to know whether any prepriming proteins are required for this reaction, extracts prepared from dnaB or dnaC mutant strains were examined. Fraction IIIs prepared from dnaB and dnaC | 8085 | Primase-dependent Initiation of Leading Strand Synthesis | 5' End of the Putative First Leading Strand—Analyses by restriction digestion described above suggest that R1 plasmid replication is initiated at a specific position on the basic replicon of R1 plasmid. In order to locate precisely the position of the initiation site, products synthesized in vitro were digested by restriction enzymes which cut the template DNA to the right of oriR, and the single strands were analyzed by denaturing polyacrylamide gel. When they were digested by Ndel or EcoRV, fragments consisting of 6–7 base ladders centered around 70 or 128 nucleotides long, respectively, were detected (Fig. 3A). Since Ndel and EcoRV cut pHM6050 at positions 2057 and 2115, respectively, the above results predicted the presence of either a specific 5' end of a nascent leading strand or 3' end of a nascent lagging strand at positions 1985–1991. | FIG. 3. 5' end of the first leading strand synthesized in vitro. A, replication products, synthesized in vitro with 0.5 μg of pHM6050 DNA in the presence of 16 μM ddTTP, were digested with Ndel (lane 1) or EcoRV (lane 2). B, replication products, prepared as in A, were digested with Ndel. Half of the digested materials were further treated with alkaline (lane 2) as described under "Materials and Methods." C, replication products, synthesized in vitro with 0.5 μg of pHM6050 DNA in the presence of various concentrations of ddTTP, were digested with Ndel. D, upper part of the gel shown in C displaying the higher molecular weight range. In all cases, the samples were analyzed on a 6% denaturing polyacrylamide gel as described under "Materials and Methods." Size markers are φX174 phage DNA digested with HaeIII (lane 3 in A, lanes 1 and 10 in C, and lane 1 in D) or pBR322 DNA-digested Map I (lane 3 in B). | FIG. 4. Strand-specific hybridization with the nascent fragment. Replication products, synthesized on pHM6050 in the 5 X strand reaction mixture with substrates containing [α-32P]TTP at 2000 cpm/pmol, were digested with EcoRV and were fractionated on an 8% denaturing polyacrylamide gel. Fragments of around 130 nucleotides were isolated and were used as a probe to hybridize with single stranded DNA (0.5 μg) spotted onto a nitrocellulose filter as indicated in the figure. Hybridization was conducted as described previously (12). |
mutants were as active as that from dnaA205 in generating RF DNAs in contrast to SS to RF conversion of ΦX174 phage DNA which was completely dependent on addition of purified proteins in both dnaB and dnaC extracts (Fig. 5). Furthermore, anti-protein i (dnaT protein) antibody, which inhibited ΦX174 replication, had no effect on replication of the chimeric phage DNA (data not shown). The above results indicate that dnaB, dnaC, and dnaT proteins are not required for the priming on this chimeric phage DNA. Therefore, a priming and elongation system composed of only SSB, primase, and pol III holoenzyme was examined. Efficient replication of M13-R101 was observed in this system, and these three purified proteins were sufficient and essential for priming and elongation on the chimeric phage (Table I). Neutral agarose gel analysis of the products synthesized in the purified system showed that form II (double-stranded nicked circular molecule) was produced (data not shown).

**Priming on the Chimeric Single-stranded Phage DNA Takes**

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>DNA synthesis (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>118</td>
</tr>
<tr>
<td>SSB</td>
<td>18</td>
</tr>
<tr>
<td>Primase</td>
<td>15</td>
</tr>
<tr>
<td>DNA polymerase III holoenzyme</td>
<td>10</td>
</tr>
</tbody>
</table>

**TABLE I**

Requirement for SS to RF replication of a chimeric phage containing the leading strand initiation site of R1 plasmid in the purified system

The standard reaction mixtures containing 0.1 μg of M13-R101 single-stranded DNA (lanes 5–8) were digested with HindIII and EcoRV (position 2115), fragments of, respectively, 180, 250, 550, or 600 nucleotides long were detected (Figs. 7A and 8). Furthermore, these fragments were shown to be derived from the lagging strand. pHM5947 contains unique HindIII and

**Fig. 5. Replication of the single-stranded chimeric phage DNA in extracts from dnaB and dnaC mutant strains.** 0.1 μg of ΦX174 phage single-stranded DNA (lanes 1–4) or M13-R101 single-stranded DNA (lanes 5–8) were replicated at 30 °C for 20 min in fraction IIs prepared from dnaB or dnaC2 strain under standard conditions (Materials and Methods), and the products synthesized were analyzed on a 0.8% agarose gel. Lanes 1, 2, 5, and 6 are assays with dnaB extract; lanes 3, 4, 7, and 8, with dnaC extract. Lanes 2 and 6, reaction mixtures supplemented with purified dnaB protein (80 ng); lanes 4 and 8, with purified dnaC protein (20 ng).

**Fig. 6.** 5’ end of the nascent DNA synthesized in vitro on the chimeric single-stranded DNA. In vitro replication products synthesized on M13-R101 (lanes 2 and 3) or on pHM6050 (lane 4) were digested with EcoRV and were analyzed on a 6% denaturing polyacrylamide gel. Lanes 2 and 4, products synthesized in fraction II from WM434 (dnaA205); lane 3, in the purified system.
positions of the recognition sites of each restriction endonuclease on pHM6050 are also shown below the gel. The boxed region and solid electrophoresis. Fragments derived from the lagging strand which are specifically terminated within oriR are indicated by arrows. The hatched region represents the oriR sequence. The boxed region and solid line represent R1 plasmid DNA and β-lactamase DNA, respectively. The hatched region represents the oriR sequence. B, 3' end of the lagging strand was determined more precisely by running the sample on a 6% denaturing polyacrylamide gel. S1 digestion generated fragments of dis-
CRETE sizes, lengths of which depended on the restriction enzyme used (Fig. 9). From the sizes of these fragments, the S1-sensitive site was deduced to be present at around position 1980, which coincides with the position of the leading strand initiation site. This result confirms the presence of a gap between the leading strand initiation site and oriR. The S1-sensitive site was readily detected in the products generated in vitro without ddTTP, indicating that a substantial fraction of in vitro products was late replication intermediates with a gap. This may suggest that the fill-in reaction of the gap is a very slow step in the replication cycle of the R1 plasmid.

**DISCUSSION**

Initiation of replication of prokaryotic replicons involves protein-DNA and protein-protein interactions at a specific sequence on the replicon, generally called replication origin (29-31). Although the nature of these interactions has been studied extensively (15, 28, 32-37), the priming sites of leading and lagging strand syntheses that result from these interactions have been examined only in a few replicons (8-10).

In this report, we have determined the site of priming for leading strand synthesis of R1 plasmid, based on the analyses of replication intermediates synthesized in vitro. When the leading strand template DNA containing the initiation site, located at nearly 400 bp downstream of oriR, was cloned onto an M13 vector, the chimeric phage required only SSB, primase, and pol III holoenzyme for replication. Furthermore, the site of priming by primase was mapped at a position identical to the leading strand initiation site. These results support the notion that leading strand synthesis of R1 plasmid is mediated only by primase at a specific site downstream of oriR. A sequence was isolated from the basic replicon of R100, a plasmid closely related to R1, that can make clear plaques when cloned into a defective M13 single-stranded phage vector.\(^2\) The vector forms only turbid plaques due to a deletion near the replication origin, and the ability of the recombinant phage to form clear plaques indicates the presence of an insert which can function as an origin for complementary strand synthesis (38). The sequence coincides with the leading strand initiation site that was mapped in this study, strongly suggesting that primase-dependent priming takes place not only in vitro but also in vivo. Primase-dependent initiation of leading strand synthesis is consistent with a previous report that primase function is essential for in vitro replication of R1 plasmid (16).

The lagging strand synthesis is terminated at a specific site within oriR. The position of the termination coincides with the 3' end of the region where RepA protein interacts with DNA (15, 28). RepA protein staying bound to oriR even after initiation of replication may simply sterically interfere with the movement of DNA polymerase and cause termination. Using similar approaches, Miyazaki et al. (39) have mapped a specific 5' end of the nascent leading strand and a 3' end of the nascent lagging strand in the replication of R100 plasmid at the same sites we report here.

Using clear plaque formation assays (38), Nomura recently isolated sequences from F and R6K plasmids that could support primase-dependent SS to RF replication in vitro. Comparison of the three plasmid-derived primase recognition sequences revealed the existence of three blocks of extensive homology.\(^4\) Limited homology was found near the priming site between the complementary strand origin of G4 phage (40) and one of the homology blocks (Fig. 8), although secondary structure found in the G4 origin was not discovered within the plasmid-derived primase recognition sequences. Yoda et al. (10) recently reported that a trinucleotide CAG frequently appears at 10-12 nucleotides upstream of primer

---

\(^2\) N. Nomura, personal communication.

RNA-DNA transition points. CAG was found at positions 1977-1979 (24), approximately 10 nucleotides upstream of the leading strand initiation site of R1 plasmid (Fig. 8).

Our data have suggested the scheme of R1 plasmid replication, as described in Fig. 10. After assembly at oriR of an initiation complex, which contains RepA protein, DNA protein, and other factors, leading strand synthesis is initiated by primase at around position 1986. Lagging strand synthesis, initiated at multiple sites, is terminated at a specific site, thus preventing further fork movement into the other direction. Once initiated, the leading strand will be synthesized continuously and the lagging strand discontinuously, with dnaB protein positioned at the replication fork to facilitate unwinding of duplex DNA (41). Okazaki fragments generated by multiple primings appear to be efficiently ligated, because no specific 5' end for a nascent lagging strand was detected by the method employed in this work. Chain elongation by pol III holoenzyme will continue until most of the DNA is replicated. The presence of significant amounts of late replication intermediates with the single-stranded DNA gap in the final products suggests that the reaction to fill in the gap is very slow: RepA protein, complexed with DNA at oriR, may stay bound until the chain elongation is almost completed and may assist decatenation of daughter molecules with its type I topoisomerase activity.3 Filling in of the gap may take place either before or after decatenation.

Among the replicons replicating through the Cairns-type mechanism, specific initiation sites for the leading strand synthesis have been identified only in CoIR1-type plasmid replication (11). The leading strand synthesis is initiated by a 555-nucleotide-long RNA primer synthesized by RNA polymerase, followed by activation of the lagging strand synthesis at an n' site located ~150 bp downstream of the RNA-DNA junction (12). The lagging strand synthesis is terminated 17 nucleotides upstream of the transition point (42, 43). In oriC or λ replication, a specific site for initiation of leading and lagging strand syntheses has not been discovered (8-10). In both cases the priming appears to occur at multiple sites on both strands, and they are located asymmetrically relative to the position of the replication origin. It is also interesting to note that the transition sites on both strands are distributed in such a way that primed DNA synthesis would cross near the replication origin (8). In bidirectionally replicating replicons, such as oriC or λ, initiation of lagging strand synthesis would automatically lead to leading strand synthesis into the other direction, if fork unwinding by dnaB protein is ensured. On the other hand, R1 plasmid, which replicates unidirectionally, would have to provide an initiation site for leading strand synthesis, since there is no lagging strand initiation to the left of oriR, that could turn into the leading strand from left to right (see Fig. 8).

As far as mechanisms of initiation of R1 plasmid replication are concerned, we still do not know how assembly of an initiation complex at oriR will lead to initiation of leading strand synthesis at nearly 400 bp downstream. Since primase can prime DNA synthesis at a specific site on a cloned single-stranded DNA but not on the double-stranded DNA containing the same sequence, it is predicted that protein-DNA and protein-protein interactions at oriR somehow destabilize duplex DNA near the leading strand initiation site to permit the recognition of the priming signal by primase. This could be achieved by unidirectional unwinding of duplex DNA by dnaB protein, as described below (Fig. 10). Studies on initiation of oriC plasmid replication in vitro suggested that dnaB and dnaC proteins may be loaded onto DNA through 13-mers, AT-rich repeating sequences present within oriC (44). Similar AT-rich repeating sequences present within oriR (28, 45) could function as an entry site for dnaB protein which then migrates on the lagging strand template while melting the

3 H. Masai, unpublished data.
duplex DNA. When dnaB protein reaches the leading strand initiation site, primase can recognize the priming signal on the exposed single-stranded DNA. Once primed, leading strand will be synthesized continuously, whereas lagging strand is primed by primase at multiple sites in conjunction with dnaB protein acting as a helicase at the replication fork (46, 47). At this point, however, we cannot rule out the possibility that assembly of an initiation complex at oriR directly induces a localized melting of duplex DNA near the initiation site without involving dnaB protein. The leading strand initiation site of R1 plasmid replication, which was identified in this study, is located outside of oriR and is obviously not essential for initiation of replication. With pHM0645 containing only the minimum basic replicon of R1 plasmid, lagging strand fragments terminating at position 1515 were still detected, whereas no specific leading strand initiation site was discovered (data not shown). In keeping with this, no specific S1-sensitive sites were detected in the late replication intermediates of pHM6045. These results suggest that DNA sequences downstream of oriR, which are required for specific initiation of leading and lagging strand synthesis, may play an important role in efficient replication of R1 plasmid. We are currently examining what region downstream of oriR affects replication of R1 plasmid both in vivo and in vitro.

R1 plasmid cannot replicate in a reconstituted oriC system supplemented with repA protein. However, addition of a partially purified fraction from crude extract restores repA-dependent replication (28). Further purification of this factor(s) will be essential for mechanistic studies of initiation and elongation of R1 plasmid replication. Termination of replication involves replication of an unreplicated region near the terminus and segregation of two daughter molecules. In R1 plasmid replication, the single-stranded gap between oriR and the leading strand initiation site should be sealed before the termination of replication. We do not know whether this is achieved by pol III holoenzyme or DNA polymerase I. Recently, type 1 topoisomerase activity has been found to be associated with purified repA protein. A possible role of repA protein in decatenation of two replicated molecules is now being examined.

Acknowledgments—We express our indebtedness to Arthur Kornberg and the members of his laboratory for the generous gift of purified E. coli replication proteins. We wish to thank Nobuo Nomura, Chikara Miyazaki, and Eiich Ohnishi for communication of results prior to publication and Tania Baker and Gerard Zurawski for critical reading of the manuscript. We also thank Gary Burget for help in preparing this manuscript.

REFERENCES

42. Minden, J. S., and Marians, K. J. (1986) J. Biol. Chem. 261, 11906-11917