Structure of nascent replicative form DNA of coliphage M13
(strain polA480ex/ribonucleotides/gaps)

SANTANU DASGUPTA* AND SANKAR MITRA†
University of Tennessee-Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Communicated by Alexander Hollanda, October 19, 1977

ABSTRACT Nascent replicative form type II (RFII) DNA of coliphage M13 synthesized in an Escherichia coli mutant deficient in the 5' → 3' exonuclease associated with DNA polymerase I contains ribonucleotides that are retained in the covalently closed RFII DNA sealed in vitro by the joint action of T5 phage DNA polymerase and T4 phage DNA ligase. These RFII molecules are labile to alkaline and RNase H, unlike the RFI produced either in vivo or from RFII with E. coli DNA polymerase I and E. coli DNA ligase. The ribonucleotides are located at one site and predominantly in one strand of the nascent RF DNA. Furthermore, these molecules contain multiple small gaps, randomly located, and one large gap in the intracistronic region.

DNA replication in coliphage M13 occurs in three distinguishable stages: (a) synthesis of the complementary strand on circular viral DNA, to produce circular replicative-form (RF) DNA; (b) a semi-conservative replication of this parental RF DNA; and (c) synthesis of single-stranded (ss) viral DNA on a circular complementary strand template and concomitant asymmetric displacement of the viral strand (1). Evidence in favor of RNA priming during the synthesis of the parental RF, either in vitro or in vivo, has been fairly well documented. Inhibition of the initiation event by rifampicin and, conversely, the lack of inhibition in extracts of mutants with rifampicin-resistant RNA polymerase (2,3) point to involvement of RNA, as does the phosphodiester linkage of deoxyribonucleotides to ribonucleotides in the RF synthesized in vitro. Furthermore, the specific requirements for covalent closure of the nicks or gaps in RFII synthesized in vitro to form the covalently closed RFII suggest the presence of an RNA segment at the 5' end of the complementary strand (2–5). Although the involvement of RNA in the initiation of DNA synthesis is a general mechanism of DNA replication in various other prokaryotic and eukaryotic systems as well (6–11), no direct evidence for RNA priming has so far been obtained for the initiation of semiconservative replication of RF DNA molecules. Rifampicin inhibition studies by Bruttig et al. (3) suggested that progeny RF synthesis involves RNA priming as well. We have confirmed these results in both a wild-type host (12) and a 5' → 3' exonuclease-deficient mutant (unpublished data). The work presented here provides additional evidence for the presence of RNA in newly replicated M13 RF by taking advantage (a) of the suggestion that the 5' → 3' exonuclease function associated with DNA polymerase I of Escherichia coli is involved in the removal of primer RNA (13, 14), and (b) of the fact that the absence of the exonuclease causes a delay in the covalent closure of the RF DNA molecules (15, 16). It therefore appeared likely that a measurable proportion of nascent RF molecules replicating in a 5' → 3' exonuclease-deficient host would contain RNA primers. We used M13 gene 5 amber mutant phage to investigate the structure of RFII during RF replication in the complete absence of progeny ssDNA synthesis (1). The results presented below show that the nascent RF DNA molecules contain multiple small gaps and one large gap, and one RNA segment in one strand. The large gap is located at a unique site in the genome.

MATERIALS AND METHODS

Escherichia coli RS 5052 (13) (E. coli polA480ex thy^-su^-), formerly referred to as polAex1, a gift from W. Masker, was used in all experiments. M13 am5H3, an amber mutant in gene 5, was kindly provided by D. Pratt. E. coli DNA polymerase I was purified according to Jovin et al. (17), and E. coli DNA ligase and the restriction endonuclease endo H-hpa II were obtained from New England Biolabs. Coliphage T5 DNA polymerase (18) was a generous gift from R. K. Fujimura, and coliphage T4 DNA polymerase and T4 polynucleotide ligase were purified according to Goulian et al. (19) and Weiss et al. (20), respectively.

A 200-ml RS5052 culture (3–4 × 10^8 cells per ml) grown at 37°C in TCG medium (21) supplemented with 0.5% casamino acid, 1 mM potassium phosphate (pH 7.4), thiamine at 5 μg/ml, and thymidine at 4 μg/ml was infected with M13 am5 (100-150 plaque-forming units per cell). After 10 min the infected culture was shifted to 42°C for 5 min before it was pulse-labeled for 1 min with [3H]thymidine (20 μCi/ml). The labeling was stopped by pouring the infected culture into 200 ml of ethanol/phenol mixture (14) at −20°C. The cells were harvested by low-speed centrifugation, were washed twice at 0°C with 0.1 M NaCl/0.05 M Tris-HCl (pH 8.0)/1 mM EDTA/0.01 M KCl, and finally were suspended in 0.05 M Tris-HCl (pH 8.0) containing 10% sucrose. The suspension was incubated at 37°C with lysozyme at 0.5 mg/ml in the presence of 40 mM EDTA for 30 min, followed by a 15-min incubation with 0.5% sodium dodecyl sulfate (NaDodSO4) and stored at 0°C overnight in the presence of 1 M NaCl. Under this condition, most of the bacterial DNA precipitates and is pelleted by short ultracentrifugation (80,000 × g, 30 min). The supernatant, containing M13 DNA, was extracted with phenol saturated with 0.2 M Tris-HCl (pH 8.0). The DNA in the aqueous phase was ethanol precipitated, suspended in 2 ml of T-E buffer (0.02 M Tris-HCl, pH 8.0/0.002 M EDTA), layered onto 36 ml of 5–20% sucrose gradient in high salt (0.02 M Tris-HCl, pH 8.0/0.002 M EDTA/1 M NaCl), and centrifuged in a Beckman SW 27 rotor

Abbreviations: RF DNA, double-stranded replicative-form DNA; RFII DNA, covalently closed RF DNA; RFIII DNA, RF DNA with one or multiple nicks or gaps in either or both strands; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; endo H-hpa II, restriction endonuclease II from Haemophilus parainfluenzae; NaDodSO4, sodium dodecyl sulfate; EdtBr, ethidium bromide; T-E buffer, 0.02 M Tris-HCl (pH 8.0)/0.002 M EDTA.

* Present address: Biophysics Laboratory, University of Wisconsin, Madison, WI 53706.
† To whom reprint requests should be addressed.

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at 25,000 rpm for 17 hr at 5°C. The peak RFI II fractions were pooled, ethanol precipitated, and suspended in T-E buffer. RFI II was further purified by a second velocity sedimentation in a low-salt 5–20% sucrose gradient (in T-E buffer) in the same way as before. This was followed by a neutral equilibrium sedimentation in CsCl, obtained by adding 5.5 ml of saturated CsCl to 1.7 ml of T-E buffer containing the DNA and centrifuging in a Beckman 40 rotor at 35,000 rpm for 36 hr at 20°C. The peak fractions were pooled, dialyzed against T-E buffer, and stored at −20°C. For some experiments, total RF DNA from the sucrose gradient in high salt was pooled and alcohol precipitated. 32P- or 14C-labeled M13 phage DNA and 3H-labeled M13 RFI DNA were prepared as described earlier (22, 23). Techniques for band sedimentation in neutral or alkaline sucrose and dye-buoyant density gradient centrifugation have also been described previously (22, 23).

The in vitro repair synthesis of RFI II was carried out following the procedure of Westergaard et al. (5). The reaction mixture contained 0.1–0.3 nmol of RFI II DNA; 20 mM Tris-HCl (pH 8.0); 10 mM 2-mercaptoethanol; 5 mM MgCl2; 10 mM (NH4)2SO4; 50 μM NAD; 20 μM each of dATP, dGTP, dCTP, and [α-32P]dTTP; 4–5 units of E. coli ligase; and 0.1–0.2 units of DNA polymerase from E. coli, T4, or T5. For sealing with T4 DNA ligase, 100 μM ATP was substituted for NAD. The reaction mixture for treatment with DNA ligase alone did not contain dNTPs. The samples were incubated at 15°C for 120 min, and the reaction was stopped with 20 mM EDTA and 0.5% Sarkosyl. Unreacted [32P]dTTP was removed by filtration in a 30-ml Sephadex G-50 column with 1 mM Tris-HCl (pH 8.0). The DNA was pooled, concentrated by evaporation at room temperature, and digested for 6–8 hr with 25 units of endo R-Hpa II at 37°C in a volume of 100–200 μl containing 10 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 3 mM diethiothreitol; EDTA was then added to 12 mM.

Electrophoresis of Hpa II-digested DNA was performed in discontinuous polyacrylamide gels (12 cm of 3% polyacrylamide at the top with 5 cm of 10% polyacrylamide at the bottom) in glass tubes (6-mm inside diameter) at 2 mA per tube for 15 hr (24). The gels were then sliced into 2-mm pieces, incubated overnight in 1 ml of HgO2 at 60–70°C, and assayed for radioactivity in ACS solvent (aqueous counting scintillant, Amersham/Searle), with suitable correction for spillover.

RESULTS

Joint Action of DNA Ligase and E. coli DNA Polymerase I Is Required for In Vitro Conversion of the Nascent RFI II to Alkali-Resistant RFI DNA. The nascent RF DNA molecules isolated during RFII replication of M13 in polA480ex host are mostly RFII DNA containing discontinuities (25). These molecules are expected to be converted in sequential steps into mature RFII DNA in a wild-type host. The nascent RFII DNA preparation should therefore contain intermediates produced in these steps, including molecules with putative RNA primers.

After treating the purified RFII DNA with DNA ligase and DNA polymerase, we monitored the formation of alkali-stable covalently closed RFII by sucrose gradient sedimentation at pH 12.5. At this pH, the RFII DNA molecules undergo complete strand separation and the linear and circular ssDNAs sediment together as a single band near the top of the gradient (Fig. 1A), separated from the faster-moving RFII. Treatment of RFII DNA with E. coli DNA ligase or T4 DNA ligase did not produce any RFII DNA (Fig. 1 B and C), implying the presence of a gap, ribonucleotide-containing sequences, or both. We should point out that T4 DNA ligase, but not E. coli DNA ligase, can ligate

![Fig. 1. Band sedimentation in alkaline sucrose of pulse-labeled M13 RFII DNA.](image-url)
erved not only with *E. coli* DNA polymerase I but also with T5 DNA polymerase (Fig. 2 A and B). Up to 30–40% of the total RFII DNA was converted into RFI with T4 DNA ligase and T5 DNA polymerase (see Table 1). However, 90–95% of this RFI is alkali sensitive (Fig. 2C, and Table 1). Treatment with *E. coli* DNA ligase and *E. coli* DNA polymerase I was almost twice as efficient in sealing the gap(s) in RFII (Table 1), and at least 70% of the resulting RFI DNA molecules survived subsequent treatment with alkali (Fig. 2D).

The RFI DNA formed by treating RFII isolated from RS 5052 with T4 DNA ligase and T5 DNA polymerase was treated with RNase H (26), and the proportion of RFI converted to RFII was examined in a dye-buoyant density gradient. Most of the RFI DNA molecules (84%) were susceptible to RNase H and were converted to RFII (Fig. 3 A and C). In contrast, as expected, 3H-labeled M13 RFII purified from infected wild-type cells was only slightly susceptible to RNase H (Fig. 3 B and D).

**Table 1. Conversion of M13 RFII into RFI by various treatments**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DNA present as RFI, %</th>
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<tbody>
<tr>
<td>NaOH-treated RF DNA after incubation with:</td>
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</tr>
<tr>
<td>T5 DNA polymerase and T4 DNA ligase</td>
<td>0–5</td>
</tr>
<tr>
<td><em>E. coli</em> DNA polymerase I and <em>E. coli</em> DNA ligase</td>
<td>40–50</td>
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<tr>
<td>RF DNA separated in CsCl/EtdBr gradient following treatment with:</td>
<td></td>
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<tr>
<td>T5 DNA polymerase and T4 DNA ligase</td>
<td>20–40</td>
</tr>
<tr>
<td><em>E. coli</em> DNA polymerase I and <em>E. coli</em> DNA ligase</td>
<td>50–60</td>
</tr>
<tr>
<td>NaOH-treated RFI DNA isolated from CsCl/EtdBr gradient after incubation with:</td>
<td></td>
</tr>
<tr>
<td>T5 DNA polymerase and T4 DNA ligase</td>
<td>5–10</td>
</tr>
<tr>
<td><em>E. coli</em> DNA polymerase I and <em>E. coli</em> DNA ligase</td>
<td>70</td>
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</tbody>
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**FIG. 2.** Conversion of M13 RFII into RFI with DNA polymerases and ligases. Pulse-labeled RFII DNA was treated with (A) T5 DNA polymerase and T4 DNA ligase and (B) *E. coli* DNA polymerase I and *E. coli* DNA ligase and subjected to equilibrium centrifugation in CsCl/EtdBr. Sedimentation was from right to left. Fractions were collected and monitored for radioactivity. RFI peaks indicated in A and B were separately pooled, freed of the dye and CsCl, and then tested for sensitivity to alkali as described in the legend to Fig. 1. C represents the band sedimentation profile to RFII from A; D, RFI from B. Other details are as in Fig. 1.

**FIG. 3.** Susceptibility of RFI DNA to RNase H. Aliquots of RFI represented in Fig. 2A (A and B) and that purified from infected wild-type cells (B and D) were treated with 2 units of RNase H and then subjected to equilibrium centrifugation in CsCl/EtdBr. A and B represent controls; C and D, the treated samples.

The Major Gap in Nascent RFII DNA Is at a Unique Position in the M13 Genome. We filled the gaps in RFII DNA molecules by repair synthesis with T4 DNA polymerase and T4 DNA ligase, digested the product with endo R-Hpa II, and separated the fragments on polyacrylamide gels. Fig. 5 shows that a measurable amount of [32P]dTTP is incorporated into all Hpa fragments, the amount of [32P] in fragment F is by far the highest.
**DISCUSSION**

The role of RNA priming in M13 parental RF synthesis has been unequivocally established (2). Our studies were directed toward detection of ribonucleotides that initiate the replication of M13 RF DNA in the absence of progeny ssDNA synthesis. We took advantage of accumulated evidence that the 5′ exonuclease associated with *E. coli* DNA polymerase I is involved in the removal of RNA primers at the 5′ ends of nascent DNA chains.

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**FIG. 4.** Band sedimentation in alkaline sucrose of pulse-labeled RF DNA purified from RS 6052. DNA samples were layered on 11.0 ml of 5–20% sucrose gradient in 0.8 M NaCl/0.2 M NaOH/2 mM EDTA in Beckman SW 41 swinging-bucket rotor tubes containing a cushion of 0.5 ml of 60% CsCl/60% sucrose (wt/vol) at the bottom. Centrifugation was at 38,000 rpm for 12 hr at 5°. Fractions were collected from the bottom and assayed for radioactivity. Sedimentation was from right to left. (A) Control RF DNA, (B) same DNA treated with *E. coli* DNA ligase, (C) RFII DNA as described in Fig. 2A. Symbols: •, [3H]-labeled; ○, [32P]- or [14C]-labeled M13 phage DNA marker. C, L, and F indicate the positions of circular, unit-length linear, and fragmented DNA, respectively.

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**FIG. 5.** Electrophoresis of endo R-Hpa II fragments of [3H]-labeled M13 RFII after gap-filling with [32P]dTTP in the presence of T4 DNA polymerase and T4 DNA ligase. The fragments identified by letters are as described in ref. 24. The Inset indicates the ratio [32P]/[3H] of the fragments after correction for variation in their thymidine content and for background.

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*E. coli* polA450ex, as opposed to the polA1 mutant (with a normal level of 3′ exonuclease activity) is nearly normal in polymerase I activity but highly deficient in the associated 5′ exonuclease activity at both permissive and restrictive temperatures (27). The nascent M13 RFII DNA synthesized in *polA450ex* host produced RF DNA labile to alkali and RNAse H when treated with T5 DNA polymerase, but not with *E. coli* DNA polymerase I, in conjunction with the appropriate DNA ligases. Because the critical distinction between the two polymerases is the absence of 5′ exonuclease in the former, the likely interpretation of our results is the lack of removal of ribonucleotide sequences, in DNA-RNA hybrids, attached to the 5′ end of DNA strands. The advantage of this approach, developed by Westergaard et al. (5), is that it is not dependent on the number and size of polyribonucleotide stretches in DNA. The denaturation products of RFII produced by phage polymerase gave rise to both circular and unit-length linear ssDNA in alkali; it therefore appears likely that ribonucleotides are present at only one region in one strand in nascent RF DNA. There are other denaturable discontinuities in such DNA, but a majority of them are DNA-DNA gaps in both strands rather than DNA-DNA nicks, because DNA ligase alone cannot seal either strand of the DNA. Nor can there be more than one DNA-RNA nick or gap because the discontinuities are closed, producing alkali-resistant unit-length strands, with T5 DNA polymerase and T4 DNA ligase.

In *E. coli*, RNA priming of DNA synthesis occurs in two distinct stages: (a) during initiation of a round of replication (28) at the origin, a rifampicin-sensitive step presumably involving host RNA polymerase, and (b) in initiation of Okazaki pieces mediated by the rifampicin-resistant RNA polymerase activity of *dnaG* protein (29) during discontinuous synthesis (30). Because M13 RF replication involves *dnaG* function (22, 31) as well as a rifampicin-sensitive step (3), both types of initiation by polyribonucleotides may also be involved in M13 RF replication. This suggests that nascent RF molecules may have more than one region with ribonucleotides, particularly in view of the fact that nascent RF contains smaller-than-unit-length fragments observed after band sedimentation in alkaline sucrose. We found that these fragments as well as unit-length linear ssDNA shown in Fig. 4A contain both virus and complementary strand sequences [as indicated by equilibrium ultracentrifugation in alkaline CsCl and self-hybridization (data not shown)]. The fact that only one region of ribonucleotides is detectable in M13 RF DNA suggests that even in the nearly complete absence of the 5′ exonuclease associated with DNA polymerase I, there is an alternative mechanism partially compensating for such deficiency. This is supported by the observation that the conversion of nascent RFII to RFII DNA occurs at a very reduced, but nonetheless significant, rate in RS6052 (25).

Our attempts to determine the location of the ribonucleotides in M13 RF on a restriction map were unsuccessful. The uptake of [32P]-labeled deoxyribonucleotides during *in vitro* substitution of ribonucleotides in RFII generated with phage DNA polymerase/DNA ligase was not confined to any one endo R-Hpa II fragment. This could result from either a random location of the ribonucleotides in RF DNA or a contaminating exonuclease associated with T4 DNA polymerase used for gap filling.

Our results were more successful in establishing the location of gaps in nascent M13 RF DNA molecules. In spite of a general uptake of labeled deoxyribonucleotides in different *Hpa* II fragments and a slightly higher background due to bacterial DNA contamination in the position of small DNA fragments,
the relatively high incorporation of deoxyribonucleotides in the F fragment of the Hpa II digest clearly indicates the location of the predominant gap. Suggs and Ray (32) showed that the M13 RFII DNA produced during progeny ssDNA synthesis also contains a gap in this region (in the viral strand). We have found a gap in nascent RF in wild-type host as well, presumably in the same location (C. Snyder, and S. Mitra, unpublished data). The site corresponds to the intracistronic region (33) and contains both the origin and terminus of parental RF synthesis (34) and of progeny ssDNA synthesis (32, 33, 35).

The authors thank Ms. C. Snyder for technical help and critical reading of the manuscript, Prof. J. Hurwitz for the gift of RNase H, and Dr. R. K. Fujinura for the gift of TS DNA polymerase. At the time of this research S.D. was a Postdoctoral Investigator supported by Subcontract 3322 from the Biology Division, Oak Ridge National Laboratory to The University of Tennessee. Oak Ridge National Laboratory is operated by Union Carbide Corporation for the Department of Energy.