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Biochemistry

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

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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 RFI DNA sealed *in vitro* by the joint action of T5 phage DNA polymerase and T4 phage DNA ligase. These RFI molecules are labile to alkali 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 vivo* or *in vitro*, 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 RFI 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 Brutlag *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 R·*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 33° 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° for 5 min before it was pulse-labeled for 1 min with [^{3}H]thymidine (20 μCi/ml). The labeling was stopped by pouring the infected culture into 200 ml of ethanol/phenol mixture (14) at −20°. The cells were harvested by low-speed centrifugation, were washed twice at 0° with 0.1 M NaCl/0.05 M Tris·HCl (pH 8.0)/1 mM EDTA/0.01 M KCN, and finally were suspended in 0.05 M Tris·HCl (pH 8.0) containing 10% sucrose. The suspension was incubated at 37° 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 ($NaDodSO_4$) and stored at 0° 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

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Abbreviations: RF DNA, double-stranded replicative-form DNA; RFI DNA, covalently closed RF DNA; RFII DNA, RF DNA with one or multiple nicks or gaps in either or both strands; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; endo R·*Hpa* II, restriction endonuclease II from *Haemophilus parainfluenzae;* $NaDodSO_4$, sodium dodecyl sulfate; EtdBr, 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.

at 25,000 rpm for 17 hr at 5°. The peak RFII fractions were pooled, ethanol precipitated, and suspended in T-E buffer. RFII 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°. The peak fractions were pooled, dialyzed against T-E buffer, and stored at −20°. For some experiments, total RF DNA from the sucrose gradient in high salt was pooled and alcohol precipitated. ^{32}P- or ^{14}C-labeled M13 phage DNA and ^{3}H-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 RFII was carried out following the procedure of Westergaard *et al.* (5). The reaction mixture contained 0.1–0.3 nmol of RFII DNA; 20 mM Tris·HCl (pH 8.0); 10 mM 2-mercaptoethanol; 5 mM $MgCl_2$; 10 mM $(NH_4)_2SO_4$; 33 μM NAD; 33 μM each of dATP, dCTP, dGTP, and [α-^{32}P]dTTP; 4–5 units of *E. coli* ligase; and 1–2 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° for 120 min, and the reaction was stopped with 20 mM EDTA and 0.5% Sarkosyl. Unreacted [^{32}P]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° in a volume of 100–200 λ containing 10 mM Tris·HCl (pH 8.0), 6 mM $MgCl_2$, 3 mM dithiothreitol; 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 H_2O_2 at 60–70°, 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 RFII to Alkali-Resistant RFI DNA. The nascent RF DNA molecules isolated during RF 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 RFI 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 RFI 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. 1*A*), separated from the faster-moving RFI. Treatment of RFII DNA with *E. coli* DNA ligase or T4 DNA ligase did not produce any RFI 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

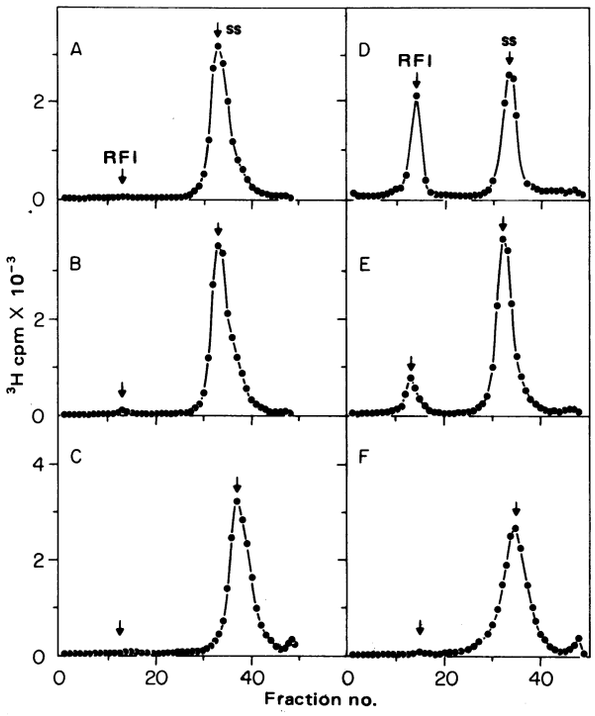

FIG. 1. Band sedimentation in alkaline sucrose of pulse-labeled M13 RFII DNA. The purified DNA was untreated (*A*) or treated with *E. coli* DNA ligase (*B*); T4 DNA ligase (*C*); *E. coli* DNA ligase and *E. coli* DNA polymerase I (*D*); *E. coli* DNA ligase and T5 DNA polymerase (*E*); or T4 DNA ligase and T5 DNA polymerase (*F*). The samples were sedimented in a 5–20% sucrose gradient in 0.8 M NaCl/0.2 M NaOH/2 mM EDTA in SW 50.1 Beckman swinging-bucket rotor tubes at 45,000 rpm for 80 min at 20°. Fractions were collected from the bottom and assayed for radioactivity. The positions of covalently closed RFI DNA and ssDNA are indicated.

DNA 3′-OH to 5′-P of RNA in dsDNA (5). When *E. coli* DNA polymerase I was used in addition to ligase, RFI was synthesized (Fig. 1*D*), indicating covalent closure of the discontinuity. Use of T5 DNA polymerase with either T4 or *E. coli* DNA ligase produced only a small amount or no alkali-stable RFI (Fig. 1 *E* and *F*). The small amount of RFI DNA produced by *E. coli* DNA ligase and T5 DNA polymerase, as opposed to the total absence of RFI with T4 DNA ligase and the phage polymerase, may be due either to more efficient sealing with the *E. coli* ligase of the fraction of RFII containing no ribonucleotides or to trace contamination of the bacterial ligase with 5′ exonuclease.

Nascent RFII DNA in *E. coli polA480ex* Contains Alkali-Labile, RNase H-Sensitive Region(s). Because T5 DNA polymerase, unlike *E. coli* DNA polymerase I, contains no 5′ exonuclease activity (18), and therefore cannot remove ribonucleotides at the 5′ terminus of DNA, the apparent absence of RFI DNA after incubation with T5 DNA polymerase in the presence of DNA ligase may result from formation of RFI containing ribonucleotides.

This possibility was tested following the procedure used by Westergaard *et al.* (5), which exploits the properties of bacterial and phage DNA polymerases and ligases. The combination of *E. coli* DNA polymerase I and *E. coli* DNA ligase should remove ribonucleotides from the 5′ end of DNA chains and produce alkali-resistant ligation, while T4 ligase with phage polymerase should preserve ribonucleotides after ligation.

When the ligase–polymerase-treated RFII DNAs were examined at neutral pH by equilibrium centrifugation in CsCl/ethidium bromide (EtdBr), formation of RFI was ob-

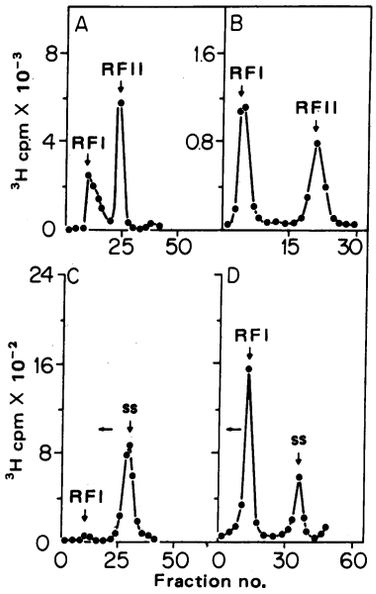

FIG. 2. Conversion of M13 RFII into RFI with DNA polymerases and ligases. Pulse-labeled RFII DNA was treated with (*A*) T5 DNA polymerase and T4 DNA ligase and (*B*) *E. coli* DNA polymerase I and *E. coli* DNA ligase and subjected to equilibrium centrifugation in CsCl/EtdBr. Sedimentation was from right 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 RFI from *A*; *D*, RFI from *B*. Other details are as in Fig. 1.

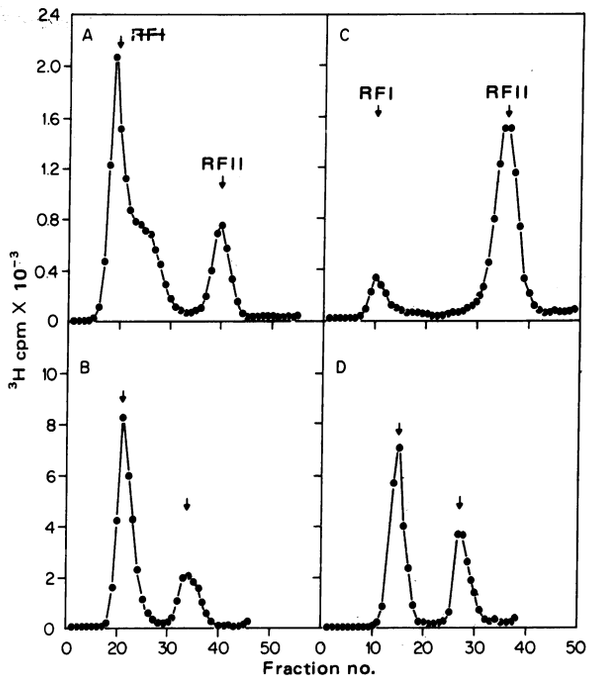

FIG. 3. Susceptibility of RFI DNA to RNase H. Aliquots of RFI represented in Fig. 2*A* (*A* and *C*) and that purified from infected wild-type cells (*B* and *D*) were treated with 2 units of RNase H and then subjected to equilibrium centrifugation in CsCl/EtdBr. *A* and *B* represent controls; *C* and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

served 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. 2*C*, 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. 2*D*).

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, ^{3}H-labeled M13 RFI 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

Experiment	DNA present as RFI, %
NaOH-treated RF DNA after incubation with:	
T5 DNA polymerase and T4 DNA ligase	0–5
E. coli DNA polymerase I and *E. coli* DNA ligase	40–50
RF DNA separated in CsCl/EtdBr gradient following treatment with:	
T5 DNA polymerase and T4 DNA ligase	20–40
E. coli DNA polymerase I and *E. coli* DNA ligase	50–60
NaOH-treated RFI DNA isolated from CsCl/EtdBr gradient after incubation with:	
T5 DNA polymerase and T4 DNA ligase	5–10
E. coli DNA polymerase I and *E. coli* DNA ligase	70

Nascent RFII DNA Contains One Ribonucleotide-Containing Region and Each Strand Contains One or More Gaps. The number of ribonucleotide-containing regions in one or both strands in the nascent RFII DNA was determined by examining the denaturation products of the alkali-labile RFI DNA [produced by treatment with T4 DNA ligase and T5 DNA polymerase (Fig. 3*A*)] on a prolonged 5–20% alkaline sucrose gradient sedimentation. Fig. 4 shows this band sedimentation profile along with that of untreated RF DNA and that of the same DNA treated with *E. coli* DNA ligase alone. The RFI DNA produced linear and circular full-length single strands in alkali, in contrast to the control RF, which yielded a considerable number of small fragments in addition to unit-length linear single strands. The RF DNA treated with *E. coli* DNA ligase alone produced a small increase in RFI but no significant increase in formation of circular single strand.

We would like to point out that the sealing of nascent RFII DNA is also retarded in the *polA1* mutant (unpublished data), which supports the earlier suggestions (14, 27) that the polymerase and exonuclease activities of DNA polymerase I act in concert. Additional evidence for concerted action of the exonuclease and polymerase activities of DNA polymerase I was provided in pyrimidine dimer excision and DNA repair experiments (27).

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 [^{32}P]dTTP is incorporated into all *Hpa* fragments, the amount of ^{32}P in fragment F is by far the highest.

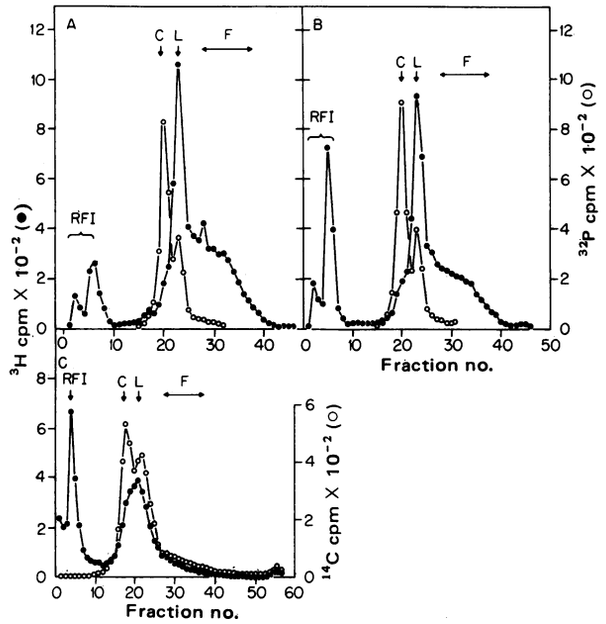

FIG. 4. Band sedimentation in alkaline sucrose of pulse-labeled RF DNA purified from RS 5052. DNA samples were layered on 11.0 ml of 5–20% sucrose gradient in 0.8 M NaCl/0.2 M NaOH/2 mM EDTA in Beckman SW 41 swinging-bucket rotor tubes containing a cushion of 0.5 ml of 60% CsCl/60% sucrose (wt/vol) at 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*) RFI DNA as described in Fig. 2*A*. Symbols: ●, ^{3}H-labeled; ○, ^{32}P- or ^{14}C-labeled M13 phage DNA marker. C, L, and F indicate the positions of circular, unit-length linear, and fragmented DNA, respectively.

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.

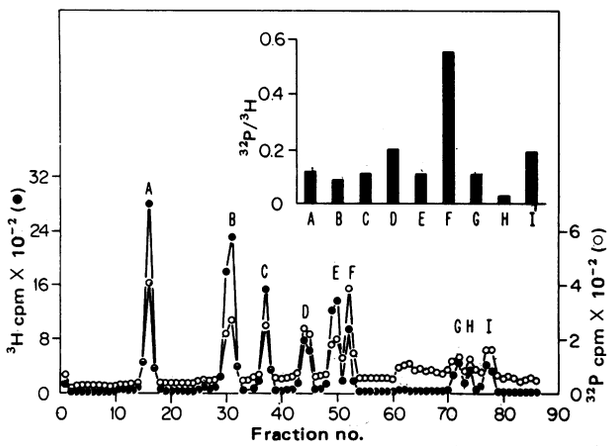

FIG. 5. Electrophoresis of endo R·*Hpa* II fragments of ^{3}H-labeled M13 RFII after gap-filling with [^{32}P]dTTP in the presence of T4 DNA polymerase and T4 DNA ligase. The fragments identified by letters are as described in ref. 24. The *Inset* indicates the ratio ^{32}P/^{3}H of the fragments after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

E. coli polA480ex, as opposed to the *polA1* mutant (with a normal level of 5′ 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 *polA480ex* host produced RFI 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 RFI 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. 4*A* 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 RFI DNA occurs at a very reduced, but nonetheless significant, rate in RS5052 (25).

Our attempts to determine the location of the ribonucleotides in M13 RF on a restriction map were unsuccessful. The uptake of ^{32}P-labeled deoxyribonucleotides during *in vitro* substitution of ribonucleotides in RFI 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).

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