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Replication of Bacteriophage M13

XV. Location of the Specific Nick in M13 Replicative Form II Accumulated in *Escherichia coli polA*ex1

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M13 replicative form II (RFII) DNA was prepared from *Escherichia coli* RS5052 (*polA*ex1) cells in the late stage of infection, and the DNA sequence at the discontinuity was examined. The data presented here suggest that the single discontinuity in the late stage of infection RFII maps at the same position as the gene *II* protein nicking site on fd RFI which was determined in vitro (Meyer et al., Nature (London) **278**:365-367, 1979) and has a 5′ terminal nucleotide sequence identical to that at the nick produced by gene *II* protein in vitro. The discontinuity in the in vivo RFII appears to be a single break in the phosphodiester backbone, leaving a 3′ OH terminus. RFII molecules containing a gap, i.e., missing nucleotides at the site of discontinuity, were not detected.

In the late stage of infection of filamentous single-stranded bacteriophages such as M13, f1, and fd, phage DNA synthesis is asymmetric, that is, the viral strand is synthesized by using the complementary strand of the duplex replicative form (RF) DNA as a template (1, 10, 17). The gene *II* protein nicks the viral strand of the RFI DNA (8) to produce the RFII molecule whose viral strand is replaced by the newly synthesized viral DNA. The 3′ end of the nicking site of the RFII is postulated to serve as the primer for initiation of viral strand synthesis by the rolling circle mechanism (9, 18). The origin-terminus of viral strand synthesis, identified with the discontinuity in the viral strand of the RFII in the late stage of infection, is located within *Hpa*II F (19) and *Hae*III G (10; J. M. Cleary and D. S. Ray, unpublished data) fragments (Fig. 1). Here we report the nucleotide sequence of the discontinuity site of the RFII accumulated in the late stage of infection.

MATERIALS AND METHODS

Strains and media. Bacterial strains, media, centrifugation techniques, and lysis of cells have been described previously (4).

Enzyme and chemicals. Restriction endonucleases *Hae*III and *Hin*fI were isolated in our laboratory (20). Restriction endonucleases *Hpa*II and *Ava*I were purchased from Boehringer Mannheim Corp. and Bethesda Research Laboratories, Inc., respectively. Polynucleotide kinase from T4-infected *Escherichia coli* was purchased from P-L Biochemicals. Bacterial alkaline phosphatase was purchased from Worthington Biochemicals Corp. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham Corp.

Preparation of M13 late stage of infection RFII DNA. *E. coli* RS5052 (*polA*ex1) cells were grown in glucose-Casamino Acids medium at 32°C to a cell density of 2×10^8 cells per ml and then infected with M13 phage at a multiplicity of infection of 50. At 60 min after infection, the temperature of the culture medium was raised to 43°C. After 15 min of incubation at 43°C, the cells were quickly chilled, harvested, and lysed. RNA was degraded by RNase A and removed by Bio-Gel A-5m column chromatography. RFI and host linear DNA were separated from RFII by ethidium bromide-cesium chloride and Hoechst 33258-cesium chloride (13; see below) equilibrium centrifugations, respectively. The yield was approximately 2 μg of RFII per liter of culture.

Hoechst 33258-cesium chloride equilibrium centrifugation. To separate M13 RF DNA from host DNA, Hoechst 33258 dye which preferentially binds to A·T base pairs (13) was used. About 400 μg of host linear DNA and M13 RF DNA was dissolved in 0.6 ml of lysing buffer (13) (0.05 M Tris-hydrochloride (pH 7.6), 0.1 M EDTA, 0.5% Sarkosyl) and 23.2 ml of H_2O. Subsequently, 1 ml of 1 mg of Hoechst 33258 per ml of water was added. The density was adjusted to 1.399 by adding 30.6 g of CsCl. After centrifugation for 40 h at 20°C and 40,000 rpm in a Beckman type 65 rotor, the upper band (M13 RF DNA) was made visible by UV illumination and recovered. The dye was removed by shaking several times with isobutyl alcohol.

Digestion of DNA with restriction endonucleases. *Hae*III, *Hpa*II, and *Hin*fI reactions contained 10 mM Tris-hydrochloride (pH 7.4), 7 mM $MgCl_2$, 7 mM β-mercaptoethanol and were incubated at 37°C. *Ava*I reactions contained 20 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 30 mM NaCl and were incubated at 37°C.

Purification of restriction fragments. After electrophoresis on polyacrylamide gels, the DNA bands were excised, and gel slices were crushed and soaked in 5 mM EDTA (pH 7.5) solution. After gentle shaking at 37°C overnight, gel debris was removed by

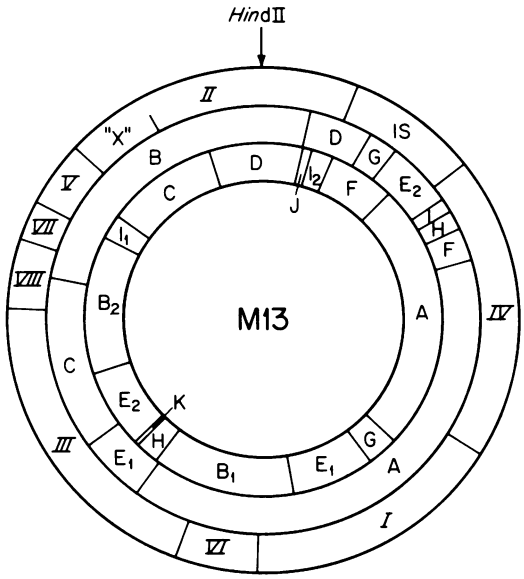

FIG. 1. *Physical and genetic map of M13 DNA. The outer circle represents the genetic map. IS denotes an intergenic space between genes II and IV. The middle and inner circles show the locations of HaeIII and HpaII restriction fragments, respectively. The arrow at the outside indicates the single cleavage site of restriction endonuclease HindII. The 5′ → 3′ polarity of the viral strand DNA is counterclockwise on this map.*

low-speed centrifugation and Sephadex G-50 column chromatography.

Kinase treatment. The kinase reactions were done by the method of Maxam and Gilbert (14).

Base-specific cleavage. The chemical reactions that produce base-specific cleavages of DNA chains were done by the method of Maxam and Gilbert (14).

One-dimensional polyethyleneimine-cellulose thin-layer chromatography. The 5′-deoxynucleotide monophosphates were separated by thin-layer chromatography with a solution of 6 g of $Na_2B_4O_7 \cdot 10 H_2O$, 3 g of H_3BO_3, and 25 ml of ethylene glycol in 70 ml of water (16).

Two-dimensional homochromatography. The purified DNA fragments were partially digested at 37°C for 20 min with 200 ng of DNase I and 100 ng of snake venom phosphodiesterase in a 20-μl reaction mixture (10 mM triethylammonium carbonate [pH 8.1], 5 mM $MgCl_2$, 5 μg of sonicated calf thymus DNA). The digestion products were separated by a two-dimensional system (3, 11, 23) which involves electrophoresis on cellulose-acetate at pH 3.5 in the first dimension and homochromatography on a polyethyleneimine-cellulose thin layer using Homomix II (11) in the second dimension.

***Hin*fI-A-*Hpa*II-large and *Hae*III-G-*Ava*I-large fragments.** *Hin*fI-A and *Hae*III-G were purified from M13 RFI DNA. After 5′ termini labeling with kinase and [γ-^{32}P]ATP, both fragments were cleaved with *Hpa*II and *Ava*I to separate two 5′ termini, respectively. Four radioactive fragments, that is, *Hin*fI-A-*Hpa*II-large, *Hin*fI-A-*Hpa*II-small, *Hae*III-G-*Ava*I-large, and *Hae*III-G-*Ava*-I-small, were purified and used as control fragments.

RESULTS AND DISCUSSION

Previous results (4, 5) indicated that *E. coli* RS5052 (*polA*ex1) cells accumulated the RFII of M13 at the restrictive temperature. Therefore, the RFII molecules were prepared from RS5052 cells in the late stage after infection with M13 wild-type phages. Since the discontinuity in the RFII at the late stage of infection was mapped within the *Hae*III G fragment (10; Cleary and Ray, unpublished data), the RFII at the late stage of infection was cut with *Hae*III restriction endonuclease and the *Hae*III G fragment was purified. After pretreatment with bacterial alkaline phosphatase, all the 5′ ends were labeled by using T4-polynucleotide kinase and [γ-^{32}P]-ATP (14). To analyze only the viral strand fragments, complementary strand DNA was hybridized to excess phage single-strand DNA before loading onto a gel. While complementary strand fragments hybridized with phage single-strand DNA were trapped at the top of the gel (data not shown), three bands (bands A, B and C in Fig. 2) derived from viral strand DNA were seen in the 15% polyacrylamide gel (14). Band A is an intact viral strand fragment of *Hae*III-G (142 bases long) and apparently comes from *Hae*III G fragments of RFII molecules having discontinuities located outside of *Hae*III-G. The sizes of bands B and C were shown to be about 86 and 54 bases long, respectively, compared with size markers. Since the intensity of bands B and C is similar, both fragments appear to be present in almost equal molar amounts. When only the 5′ end of the discontinuity site of the RFII at the late stage of infection was labeled with kinase before *Hae*III restriction endonuclease digestion, a single band corresponding to band B was detected (data not shown). These results suggest that there is one predominant discontinuity within the *Hae*III-G viral strand of the RFII at the late stage of infection and that the discontinuity is located between the 3′ end of band C and the 5′ end of band B, i.e., about 54 bases downstream (to the right in Fig. 4) from the *Hae*III-E_2-*Hae*III-G junction on the viral strand. The RFII molecules at the late stage of infection which have discontinuities located outside of *Hae*III-G may be produced by random nicking during preparation or by a decreased ability of DNA polymerase I of RS5052 to repair the excision site of misincorporated dUTP (24).

To localize bands B and C of Fig. 2 precisely, both bands were purified from a gel and 5′ terminal sequences were examined. The 5′ end

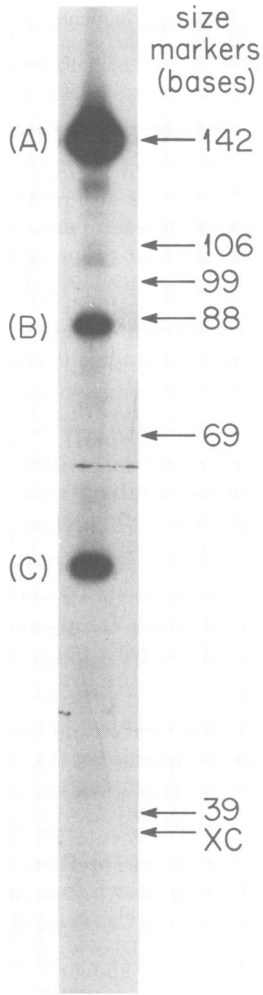

FIG. 2. *Gel electrophoresis of the HaeIII G viral strand fragments of the late stage of infection RFII. Three picomoles (11 μg) of the purified late stage of infection RFII was cut with HaeIII, and 1 pmol of the HaeIII-G was recovered. After pretreatment with alkaline phosphatase, the HaeIII G fragment was denatured at 100°C for 3 min and labeled by using 10 units of T4-polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol) in a 100-μl reaction mixture by the method of Maxam and Gilbert (14). Fifty picomoles of M13 phage single-stranded DNA was added to the labeled HaeIII G fragments, and hybridization was carried out at 65°C for 2 h in a 50-μl reaction mixture (1 M triethylammonium carbonate, pH 8.1). The sample was desalted four times by freeze-drying and was loaded onto a 15% polyacrylamide–7 M urea gel. Although 7 M urea was contained in this gel, double-stranded DNA fragments were not denatured if heat treatment and addition of alkali before loading were omitted (data not shown). The arrows show the position of xylene cyanol (XC) dye and of several single-stranded DNA size markers whose sizes are given in terms of the number of bases.*

of band B was found to be deoxyadenosine; by polyethyleneimine-cellulose thin-layer chromatography analysis (16) of the complete digests with DNase I and snake venom phosphodiesterase (data not shown). Therefore, probable structures of band B were restricted to the three alternatives, that is, 5′ AATAG-----TTCGG 3′ (88-mer), 5′ ATAGT-----TTCGG 3′ (87-mer) or 5′ AGTGG-----TTCGG 3′ (85-mer) (Fig. 4). To determine the 5′ terminal sequence of band B, the fragment was partially digested with DNase I and snake venom phosphodiesterase, and the products were separated by a two-dimensional system (Fig. 3a) (3, 11, 23). As the mobility shift between the first three spots corresponding to mono-, di-, and trinucleotides did not determine a unique sequence, a marker fragment, *Hin*fI-A–*Hpa*II-A-large fragment, of which the 5′ terminal sequence is 5′ AATCTTT-----3′ (P. van Wezenbeek, T. Hulsebos, and J. G. G. Schoenmakers, submitted for publication), was also partially digested and separated by a two-dimensional system (Fig. 3c). Compared to the pattern of *Hin*fI-A–*Hpa*II-large fragment (Fig. 3c) and considering the limitations of this system (data not shown), the shift shown in Fig. 3a is consistent with the sequence 5′ A(A or G)TAG-----3′. Although 5′ AATAG 3′ has been found at 5′ termini of one of three probable oligomers, 5′AGTAG 3′ does not exist in the viral strand of *Hae*III-G. These results, therefore, suggest that the band B fragment is 5′ AATAG-----TTCGG 3′ (88-mer) (Fig. 4).

The 5′ terminal sequence of band C was also examined. The 5′ end of band C was found to be deoxycytidine (data not shown). Compared to the pattern (Fig. 3d) of a marker fragment *Hae*III-G–*Ava*I-large fragment, of which the 5′ terminal sequence is 5′ CCATCG-----3′ (20), the pattern of band C shown in Fig. 3b suggests the sequence 5′ C(C or A)(C or A or G)TCG-----. Among several possible sequences (5′ CCCTCG 3′, 5′ CACTCG 3′, etc.), only 5′ CCATCG 3′ was found in the *Hae*III-G viral strand and was mapped at the 5′ end of the *Hae*III G fragment (Fig. 4). These data suggest that the band C fragment has come from the 5′ proximal region of the *Hae*III-G viral strand. Also, the size of band C has been determined precisely, compared with sequencing patterns (14) of *Hae*III-G–*Ava*I-large fragment (Fig. 5). Figure 5 shows that band C migrates between 54-mer and 55-mer. As dephosphorylation causes a reduction in migration by half (22; unpublished data) of one nucleotide interval and 54-mer and 55-mer have phosphates at the 3′ ends, the results of Fig. 5 suggest that the size of band C is 54 bases and the 3′ end of band C is hydroxyl. Recently, Meyer et al. (15) reported that gene *II* protein

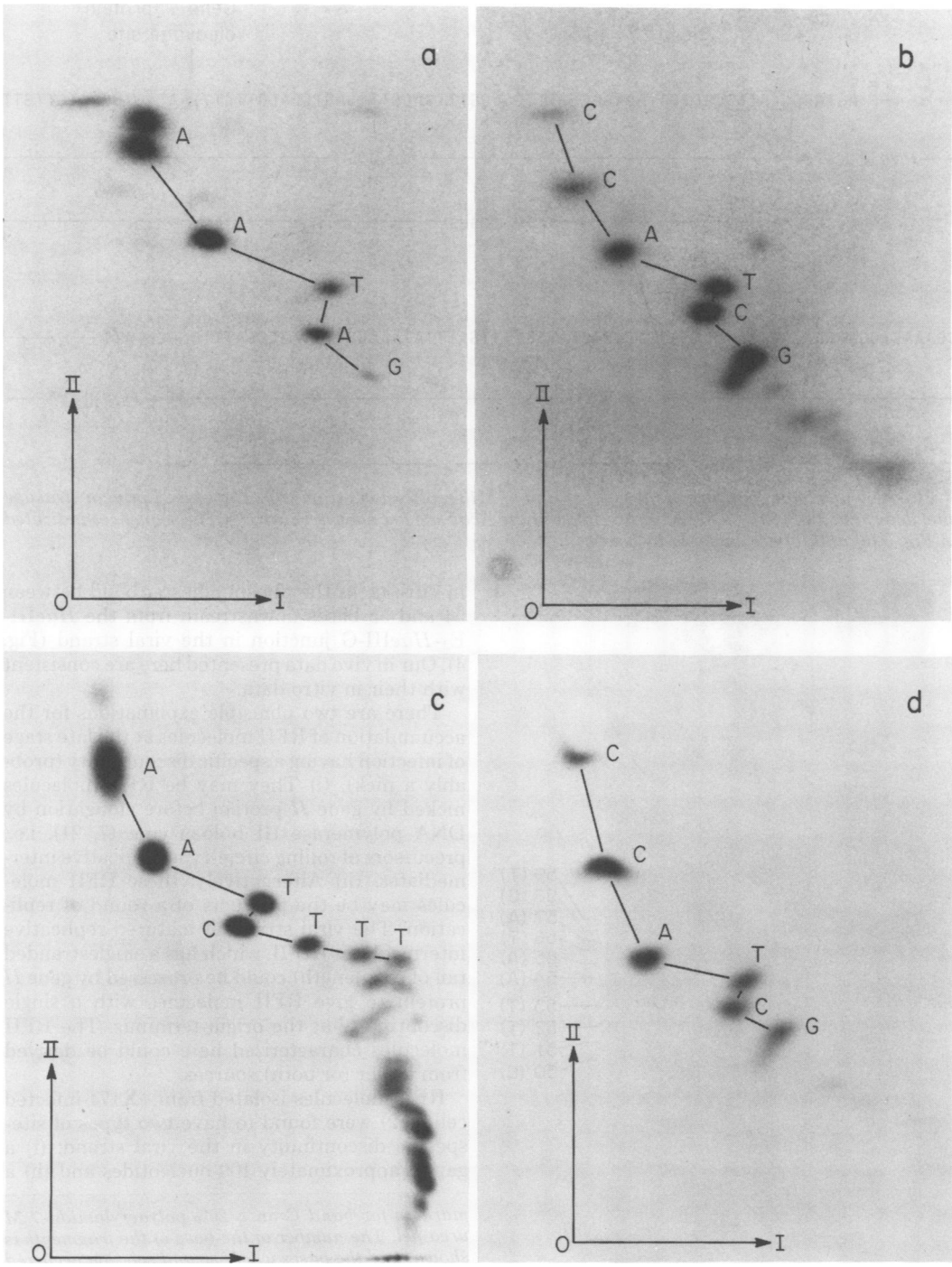

FIG. 3. *Two-dimensional homochromatograms of a partial digest of (a) band B of Fig. 2; (b) band C of Fig. 2; (c) HinfI-A–HpaII-large fragment; and (d) HaeIII-G–AvaI-large fragment. The first dimension (I) was electrophoresis on cellulose-acetate at pH 3.5. The second dimension (II) was homochromatography on polyethyleneimine-cellulose thin layer. The most likely nucleotide causing the shift is indicated in (a) and (b). The nucleotide causing the shift is indicated according to the sequencing data (see text) in (c) and (d). O indicates the origin. The reason for two spots existing at the position corresponding to a mononucleotide in (a) is unclear.*

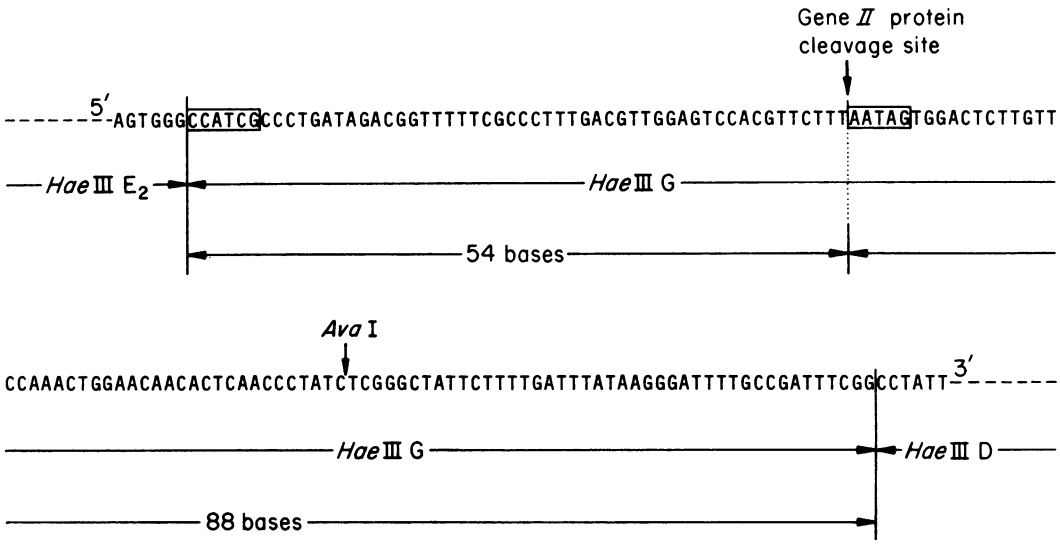

FIG. 4. *Nucleotide sequence of the viral strand of M13 replication origin (20). The gene II protein cleavage site, determined in vitro by Meyer et al. (15), and the AvaI site are shown by arrows. The sequences indicated in Fig. 3 (a) and (b) are enclosed by boxes.*

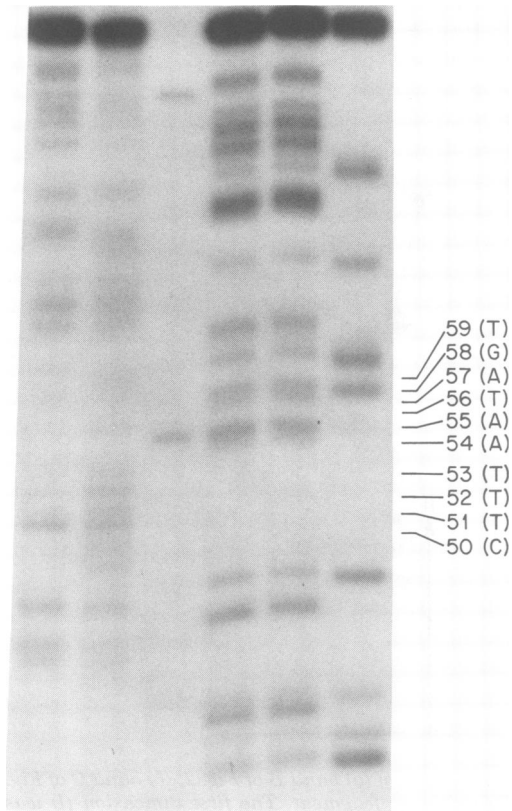

FIG. 5. *Electropherogram of band C of Fig. 2. HaeIII-G-AvaI-large fragment was treated by the method of Maxam and Gilbert (14) and run as size markers for band C on a 20% polyacrylamide-7 M urea gel. The number of the base of the fragments is shown, and the bases where strand scission occurred are indicated in parentheses. From the left lane, C, C+T, bands (B) and (C) of Fig. 2, A+G, A+G, G. As bands (B) and (C) which were mixed with "A+G" in the fourth lane from left are not visible, the lane has also been indicated as "A+G" in addition to the fifth lane from left where only "A+G" was loaded.*

in vitro nicks the phosphodiester bond between 54 and 55 bases downstream from the *Hae*III-E_2-*Hae*III-G junction in the viral strand (Fig. 4). Our in vivo data presented here are consistent with their in vitro data.

There are two plausible explanations for the accumulation of RFII molecules at the late stage of infection having a specific discontinuity (probably a nick). (i) They may be RFII molecules nicked by gene *II* protein before elongation by DNA polymerase III holoenzyme (7, 21), i.e., precursors of rolling circle-type replicative intermediates. (ii) Alternatively, these RFII molecules may be the products of a round of replication. The viral strand of matured replicative intermediates (RFII, which has a singlestranded tail of unit length) could be processed by gene *II* protein to give RFII molecules with a single discontinuity at the origin-terminus. The RFII molecules characterized here could be derived from either (or both) sources.

RFII molecules isolated from φX174-infected cells (12) were found to have two types of site-specific discontinuity in the viral strand: (i) a gap of approximately 100 nucleotides and (ii) a

nick or small gap. The M13 RFII molecules described here apparently correspond to this latter class of φX174 RFII. M13 molecules having such extensive gaps would not have been analyzed in our procedure, which involved the isolation of a fragment migrating at the rate of the M13 *Hae*III G fragment. Therefore, it is not clear whether there are such molecules, also, in M13-infected cells. Eisenberg et al. (6) reported φX174 RFII molecules whose gap sizes in the viral strand were 12 to 16 nucleotides. These molecules may have arisen as a result of exonucleolytic degradation at the specific nick in the RFII as has been observed by others (2). Since this degradation may involve the 5′ exonuclease activity of polymerase I, our lack of detection of a similar class of RFII may reflect the defective 5′ exonuclease activity of polymerase I in the *E. coli* mutant used in our experiments.

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