Proc. Nat. Acad. Sci. USA
Vol. 71, No. 3, pp. 984–988, March 1974

Structure of Nascent φX174 Replicative Form: Evidence for Discontinuous DNA Replication*

(DNA polymerase/restriction nuclease/competition hybridization/gap-filling/*polA*)

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Communicated by S. E. Luria, November 16, 1973

ABSTRACT **φX174 RF (replicative form) II DNA, labeled *in vivo* with [*methyl*-^{3}H]thymidine, was isolated from *Escherichia coli polA* (DNA polymerase I-deficient) and *polA*$^+$ cells during RF replication. [^{32}P]dCMP was incorporated into the gaps present in the RF II DNA with [α-^{32}P]dCTP and T4 DNA polymerase. Sedimentation in alkaline sucrose gradients revealed that much of the incorporated ^{32}P was present in a heterogeneous collection of fragments shorter than unit length. Inclusion of polynucleotide ligase in the gap-filling reaction increased the average size of the ^{32}P-labeled fragments. Gel electrophoresis of the products formed by digestion of the ^{32}P-labeled RF II molecules with the restriction nuclease, endonuclease R, indicated that in the population of RF II molecules gaps could occur anywhere in the genome. Competition-annealing experiments provided evidence that the majority of the label incorporated into gaps was present in the minus strand. RF II molecules isolated from *polA*$^+$ cells were enriched for gaps in a unique region of the genome in comparison with RF II molecules isolated from *polA* cells. The presence of multiple gaps in the minus strand implies that it is synthesized by a discontinuous mechanism during φX RF replication.**

The life cycle of bacteriophage φX174 in *Escherichia coli* comprises three distinct phases. The first is the synthesis of a minus strand complementary to the viral plus-strand template, resulting in the production of a double-stranded circular parental replicative form (RF) molecule. A study of this event *in vitro* has shown that a polyribonucleotide must first be synthesized to act as a primer for the appropriate DNA polymerase (1). In the second phase, between 3 and 20 min after infection under normal conditions, RF replication ensues and some 15–30 double-stranded progeny RF molecules accumulate in the cell (2). It is this phase we are concerned with here. The third and final phase is the asymmetric replication of the RF to produce, ultimately, circular single-stranded progeny DNA. The immediate precursor of the mature viral DNA is probably a linear strand, shorter than unit length, that is converted to a circular molecule by the combined action of DNA polymerase and DNA ligase (3).

Throughout the φX174 life-cycle two distinct RF structures can be identified. RF I is a covalently-closed circular duplex which *in vitro* assumes a superhelical configuration. RF II is a circular duplex with at least one discontinuity in one of the strands; this molecule seems to be the initial product of RF replication. The discontinuity (or discontinuities) in the majority of the nascent RF II molecules has been shown to be a gap, since nucleotides must be incorporated into the RF before ligase can convert the RF II to RF I (4). In this paper we present a study of the location and distribution of these gaps in the RF II molecules produced during RF replication.

Abbreviation: RF, replicative form.

* This is paper no. 8 in the series "The Mechanism of Replication of Single-Stranded φX174 DNA." Paper no. 7 is ref. 3.

MATERIALS AND METHODS

Organisms. *E. coli* C, *E. coli HF 4720 polA*1 and its *polA*$^+$ revertant (D88) have been described (4). φX174*am*3 is a lysis-defective cistron E mutant of φX.

Reagents and Enzymes. [*methyl*-^{3}H]Thymidine (40–60 Ci/mmol) was purchased from New England Nuclear Corp. [α-^{32}P]dCTP was prepared by the procedure of Symons (5). Pronase, grade B, was bought from Cal Bio Chem. T4 DNA polymerase was purified through the hydroxylapatite stage (6). T4 polynucleotide ligase was purified as described by Weiss (7). Endonuclease R was purified from *Hemophilus influenzae* according to Smith and Wilcox (8), except that the nucleic acids were removed with streptomycin sulfate rather than an agarose column. Neutral sucrose gradients (5–20%, linear) contained 1 M NaCl, 50 mM Tris·HCl, pH 7.5, and 2 mM EDTA in addition to the sucrose.

Preparation of RF II DNA. Four 500-ml cultures of the appropriate *E. coli* strain were grown in mT3XD (9) to about 4×10^8 cells per ml and infected with φX*am*3 at a multiplicity of infection of 5. Six minutes after infection, [^{3}H]thymidine was added (1 μCi/ml); 3 min later, the cells were diluted 1:1.6 into a solution containing 75% ethanol, 20 mM sodium acetate, pH 5.5, 4 mM EDTA, 2% phenol (saturated with 0.05 M sodium tetraborate) (10). The cells were collected by centrifugation, resuspended in 160 ml of 50 mM Tris·HCl, pH 8, and incubated with 0.7 mg/ml of lysozyme and 10 mM EDTA at 37° for 3 min. Dodecyl sulfate (Na^+ salt) and Pronase (20 mg/ml, preincubated at 37° for 60 min) were subsequently added to final concentrations of 1% and 0.8 mg/ml, respectively, and the incubation at 37° continued for another 8–12 hr. *E. coli* DNA was then precipitated at 0° with 1 M NaCl–0.8% dodecyl sulfate, the supernatant was extracted with phenol, and the viral nucleic acids were precipitated with sodium acetate–isopropanol as described by Schekman *et al.* (4). The φX RF was further purified by centrifugation through a neutral sucrose gradient (SW 27 rotor, 15 hr, 25,000 rpm, 15°); 1-ml fractions were collected from the top of the gradient with a Buchler Auto-Densi-Flow. The ^{3}H-labeled 16S RF II DNA fractions were pooled, precipitated with sodium acetate–isopropanol, and banded in a

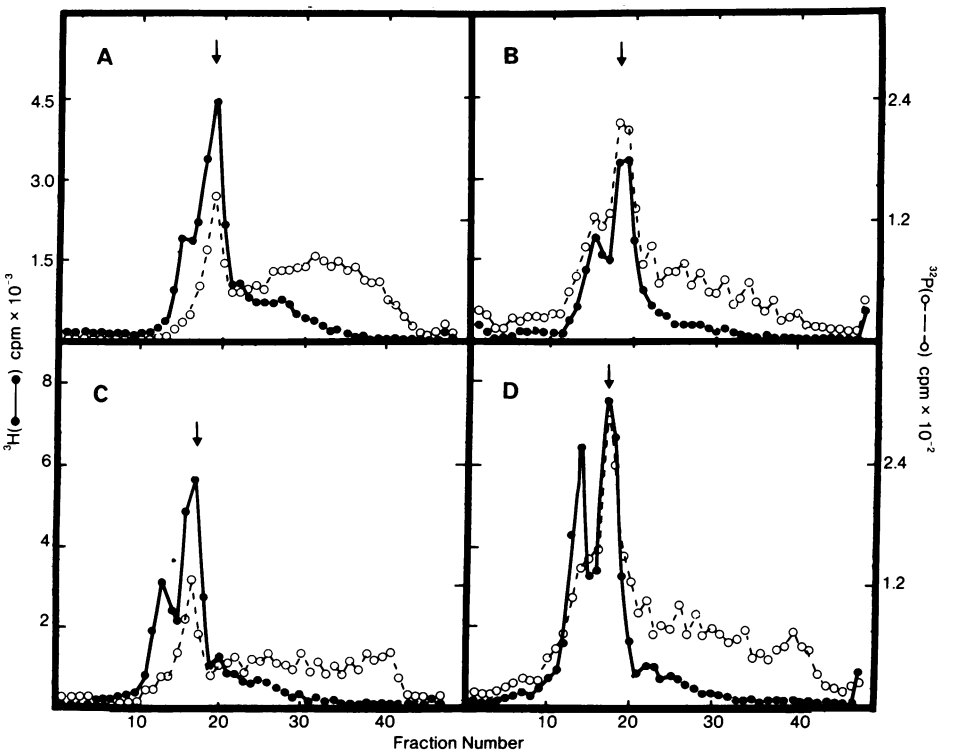

FIG. 1. Alkaline sucrose gradients of the ^3H,^{32}P-labeled RF II. The gaps in RF II [^3H]DNA were labeled with [^{32}P]dCMP as described. The doubly labeled RF II DNA was denatured with alkali and centrifuged on a linear 5–20% alkaline sucrose gradient containing 0.2 M NaOH, 0.8 M NaCl, 2 mM EDTA, 0.1% Sarkosyl, and sucrose. Centrifugation was performed in an SW 50.1 rotor for 4 hr at 50,000 rpm and 15° in an L265B centrifuge. The sedimentation profiles of the denatured RF II DNA isolated from *E. coli* HF 4720 and that reacted either with T4 DNA polymerase alone or with T4 DNA polymerase plus ligase are shown with the same scales in panels *A* and *B*, respectively. The profiles, plotted with the same scales, in panels *C* (DNA polymerase only) and *D* (DNA polymerase + ligase) represent DNA that reacted similarly, but isolated from the *polA*$^+$ revertant of HF 4720. ^3H, ●——●; ^{32}P, O- - -O. The *arrow* marks the position of φX174 unit-length DNA. Sedimentation is from right to left.

neutral CsCl density gradient (1.71 g/ml) to remove contaminating RNA. This procedure results in RF II that is pure according to the several criteria outlined in the *Results* section.

Labeling of the Gaps in RF II. The RF II [^3H]DNA (in 50 mM Tris·HCl, pH 8) was diluted into a reaction mixture containing (final concentrations) 12–30 μg/ml of RF II, 14 μM each dATP, dGTP, dTTP, and [α-^{32}P]dCTP, 17 mM mercaptoethanol, 6.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 50 mM Tris·HCl, pH 8, and 0.1 units/ml of T4 DNA polymerase in a total volume of 150 μl. The reaction was allowed to proceed at 37° for 90 min, a period sufficient to maximally label the RF II. Unless indicated otherwise, T4 polynucleotide ligase was included in the reaction at 6 units/ml, the mercaptoethanol was replaced with 10 mM dithiothreitol, and the reaction was supplemented with 66 μM ATP. Reactions were terminated by addition of EDTA to a final concentration of 25 mM. The doubly labeled RF II was repurified either by centrifugation on neutral sucrose gradients (SW 50.1 rotor, 3 hr, 50,000 rpm, 15°) or by filtration on a Bio-gel A-1.5 (Bio-Rad) column (1.6 × 30 cm) with elution buffer containing 50 mm Tris·HCl, 200 mM NaCl, 20 mM EDTA (pH 7.5). The DNA is eluted in the void volume.

RESULTS

Size of the DNA Labeled When the Gaps are Filled. φX DNA was labeled *in vivo* with [^3H]thymidine from 6 to 9 min after infection of *polA* cells (DNA polymerase I-deficient) and *polA*$^+$ cells. The RF II was purified and [^{32}P]dCMP was incorporated into the gaps in the RF II as described in *Methods*. That the RF II was uncontaminated to a detectable extent with host nucleic acids was indicated by neutral sucrose gradients showing that the ^{32}P and ^3H cosediment at 16S, by DNA·DNA hybridization (data not shown), and by the experiment with endonuclease R described below. The ^{32}P is incorporated into gaps that are characteristic of nascent RF II (4), and it is this doubly-labeled RF that is the subject of the investigations reported here. *In vivo*, under normal conditions (*polA*$^+$), the gaps are filled in fairly quickly and the RF II is converted to RF I, the final product.

When the ^{32}P,^3H-labeled RF II was centrifuged on alkaline sucrose gradients, a considerable amount of the ^{32}P-labeled DNA sedimented more slowly than unit-length φX DNA, the position of which is marked by most of the ^3H-labeled DNA. These gradients, in which circular and linear strands were not well resolved because of the high ionic strength, are presented in panels A (*polA*) and C (*polA*$^+$) of of Fig. 1. When the RF II was isolated from the *polA* mutant, a higher proportion of the total ^{32}P sedimented more slowly in comparison with RF II isolated from *polA*$^+$ cells. When T4 polynucleotide ligase was included in the reaction mixture together with T4 DNA polymerase, the sedimentation patterns shown in panels B (*polA*) and D (*polA*$^+$) of Fig. 1 were obtained. In both cases ligase caused an increase in the average sedimentation coefficient of the [^{32}P]DNA so that a larger proportion of it sedimented with φX unit-length DNA.

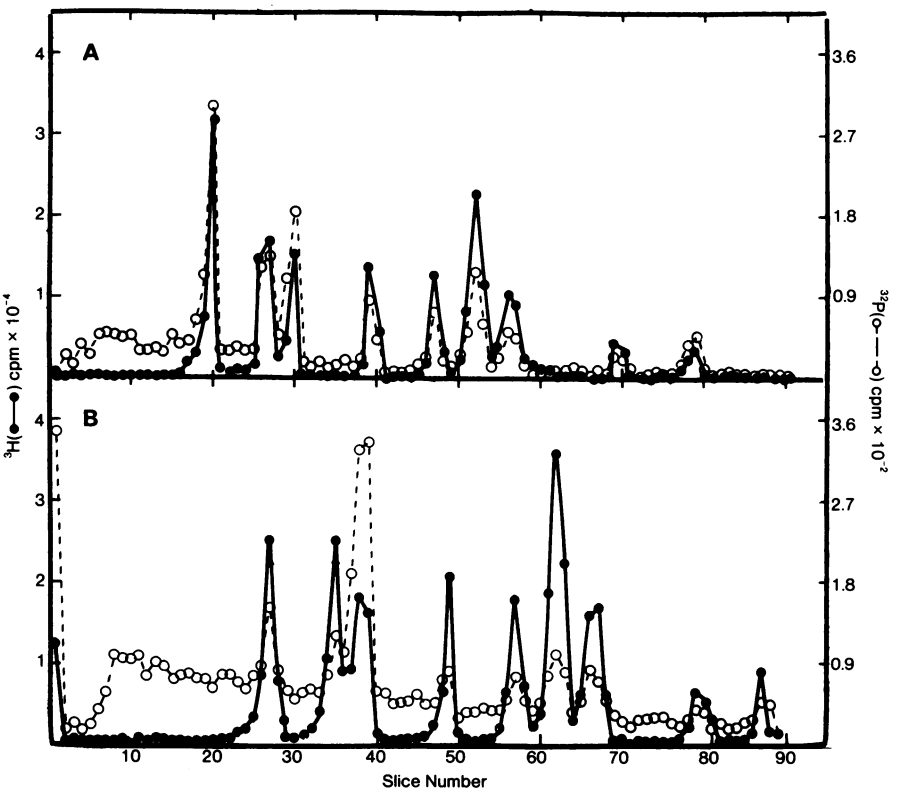

FIG. 2. Agarose–polyacrylamide gel electrophoresis patterns of an endonuclease R digest of ϕX RF II. RF II DNA was purified, the gaps were labeled as described, and the doubly labeled DNA was digested with endonuclease R. Digestion with the endonuclease, preparation of the gels, and electrophoresis were performed according to the procedures described by Edgell *et al.* (11). The gels were sliced with a Gilson Aliquogel unit; the slices were incubated at 45° for 3–6 hr in 0.4 ml of 25% H_2O_2–0.1 M NaOH, then neutralized with 2 N HCl and counted in 15 ml of Triton X-100 scintillation fluid (12). (*A*) RF II isolated from HF 4720. (*B*) RF II isolated from *E. coli* C(*polA*$^+$). ^{3}H, ●——●; ^{32}P, ○– – –○. Migration is towards the right. The peaks are designated R 1 through R 9, starting from the *left*.

About 10–20% of the RF II was converted to the covalently-closed circular duplex (RF I) and is not seen in these gradients because it has sedimented to the bottom of the tube. These results suggest that the deoxynucleotide chains containing the radioactive label inserted when the gaps are filled in are heterogeneous in size and frequently shorter than unit-length.

Location of the Gaps in the Genome. In order to determine the region(s) of the genome in which the gaps are found, the ^{32}P,^{3}H-labeled RF was digested with the restriction nuclease of *Hemophilus influenzae*, endonuclease R. This enzyme breaks ϕX RF into 12 discrete fragments, which can be resolved into 9 or 10 bands by electrophoresis on acrylamide gels (11); a number of these fragments have been shown to carry particular genetic markers. Plots of the distribution of radioactivity in acrylamide gels after electrophoresis of the fragments generated by endonuclease R digestion of the ^{32}P,^{3}H-labeled RF II are shown in panel A (*pol*A) and panel B (*pol*A$^+$) of Fig. 2. The percent of the radioactivity found in each size class of fragments is given in Table 1. For a uniformly distributed label, as to a first approximation the ^{3}H should be, the fraction of the label in each size class is proportional to the size of the fragment except in those cases (bands R6 and R7) where more than one fragment of that size is present. It is evident, first, that all fragments contain some ^{32}P and, second, that the amount of ^{32}P label in the R3 fragment is enhanced relative to the others, especially in the RF II isolated from *pol*A$^+$ cells. The fact that 60–90% of the ^{32}P is recovered in fragments characteristic of ϕX RF DNA proves that the gaps that are being filled in by T4 DNA polymerase are contained in ϕX RF II DNA. The observation that all the RF fragments contain ^{32}P label is evidence that the gaps in nascent RF II molecules can occur in many regions of the genome.

Strand Distribution of the Gaps. The question whether gaps were present in both strands of the nascent RF molecules (presumably not in both strands of any one molecule because only one strand of the semiconservatively replicated RF is newly synthesized) was investigated by a competition-renaturation procedure. In this experiment, the ^{32}P,^{3}H-labeled RF was sonicated, denatured, and annealed in the presence of various amounts of competing, unlabeled plus-strand DNA. The proportion of label that could be prevented from renaturing by competition with unlabeled plus-strand DNA was quantitated by its sensitivity to a nuclease specific for single-stranded DNA (13).

The results of this experiment, and a more detailed description of the methodology, are presented in Fig. 3 along with several controls. It is apparent that in both *pol*$^+$ and *pol*A strains, considerably more than 50% of the ^{32}P-labeled DNA remains resistant to the nuclease; if the gaps were uniformly present in plus and minus strands, then we would expect only 50% of the label to be resistant to the nuclease in the presence of excess competing plus strands. We infer from these data that there is an asymmetry in the distribution of the gaps

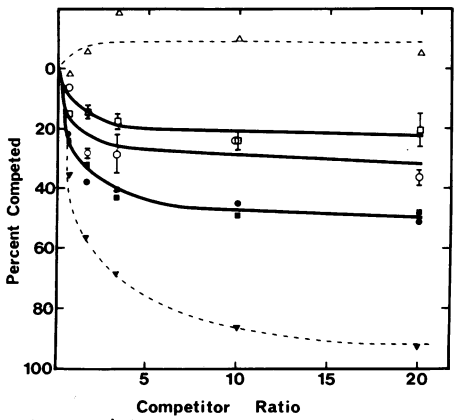

FIG. 3. Competition-annealing of the doubly labeled RF II. The $^3H,^{32}P$-labeled RF II was combined with unlabeled RF II and sonicated (Artek Sonic Dismembrator, microprobe, setting of 50 for 60 sec) in 0.25 ml of 10 mM NaCl, 10 mM Tris·HCl, pH 7.6, 0.2 mM EDTA at a concentration of 8–10 μg/ml of DNA. Unlabeled viral plus strands were similarly sonicated. Aliquots containing 0.2–0.3 μg of the RF were combined with different amounts of single-stranded DNA in 100 μl containing (final concentration) 0.1 M NaCl, 0.1 M Tris·HCl, pH 7.6, 0.1 mM EDTA. The DNA was denatured by heating at 100° for 5 min and then incubated at 60° for 24–48 hr (sufficient to give a C_0t of 1 and to reanneal over 95% of the input RF). $MgCl_2$ and *Neurospora crassa* single-strand-specific exonuclease (13) were added to concentrations of 10 mM and 1 unit/ml, respectively, and incubated at 36° for 4 hr. Aliquots were taken onto 1-inch squares of Whatman no. 5 paper; the papers were washed with 5% trichloroacetic acid, dried, and counted in a scintillation counter. The *broken lines* show controls where the RF contained label only in the plus (▼) or the minus (△) strand. The *solid symbols* are the ^3H label; the *open symbols* are the ^{32}P label. The *squares* represent RF from HF 4720 *pol*A; the *circles* represent RF from a *pol*A$^+$ strain. The experiments with the *pol*A and *pol*A$^+$ strains were done in duplicate; the points plotted are the average of the two measurements, whose ranges are indicated by the *bars*.

since only 20% (*pol*A) to 35% (*pol*A$^+$) of the ^{32}P incorporated into the gaps can be "competed out" by unlabeled plus strands. Thus, most of the ^{32}P, particularly in the RF isolated from *pol*A cells, appears to be in gaps that are present in the minus strand.

DISCUSSION

From these experiments we conclude that (*i*) in the RF molecules with gaps there are often several gaps (Fig. 1) in the strand containing them; (*ii*) the gaps can occur in many places (Fig. 2) in the genome; and (*iii*) the majority of the gaps are in the minus strand (Fig. 3), particularly in the RF II molecules isolated from the *pol*A mutant. The demonstration that incubation with polynucleotide ligase during the reaction with DNA polymerase results in an increase in the length of the ^{32}P-labeled strands shows that there must be several strands shorter than unit length aligned on the template.

Furthermore, from the facts that (*i*) 20% and 35% of the ^{32}P label is found in the plus strands derived from the RF II synthesized in *pol*A and *pol*A$^+$ cells, respectively, (Fig. 3) and (*ii*) 16.5% and 33% of the label is found in the R3 fragment derived from RF II synthesized under either *pol*A or *pol*A$^+$ conditions (Table 1), we expect that most of the plus-strand

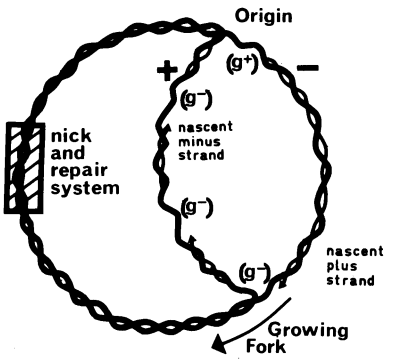

FIG. 4. Model of a φX replicating intermediate. The *thick solid lines* represent the parental strands; the *thin lines*, the newly synthesized progeny strands. In order to produce RF II molecules containing gaps of the kind we have identified above, it is necessary for the minus strand to be synthesized discontinuously and the plus strand continuously from a unique origin. The origin is in the region of cistron A (15) and the growing fork is in the region of cistrons D or E, as represented here. The gaps in the minus strand (g^-) can occur apparently anywhere, whereas the gaps (g^+) in the plus strand appear restricted to the region of cistron A. In order for the parental strands to be separated, it is necessary to envision a "nick-and-repair" system not unlike that postulated for mitochondrial DNA replication (20).

label (^{32}P) will be found in the R3 fragment. This R3 fragment contains (14) nucleotide sequences characteristic of the cistron A region, the region where a round of replication is believed to be initiated in a clockwise and unidirectional fashion (15). Also, Johnson and Sinsheimer (16) have shown an enrichment for gaps in the R3 fragment derived from RF during single-stranded DNA synthesis.

We tentatively wish to interpret our data in terms of a replicating intermediate like that shown in Fig. 4. As mentioned above, one specific prediction, currently under investi-

TABLE 1. *Distribution of radioactive label among the fragments produced by digestion of φX RF DNA with the endonuclease R of* Hemophilus influenzae.

	RF/*pol*A				RF/*pol*A$^+$			
	Percent ^3H		Percent ^{32}P		Percent ^3H		Percent ^{32}P	
Band	1	2	1	2	3	4	3	4
R1	21	20	23	29	14	21	16	18
R2	16	16	14	17	13	15	13	10
R3	10	10	17	16	13	11	34	32
R4	9	10	9	8	11	10	7	8
R5	8	8	6	6	9	8	6	8
R6	19	19	13	11	21	19	10	11
R7	10	10	9	7	12	10	9	9
R8	4	4	4	3	4	4	2	3
R9	3	3	3	3	3	2	2	1

The numbers in the Table give the percent of the radioactivity observed in each of the bands after electrophoresis in acrylamide gels of RF digested with endonuclease R. Data from four experiments are summarized. In experiments *1* and *2* the RF was isolated from *E. coli* HF 4720; in experiments *3* and *4* the RF was isolated from *E. coli* C and *E. coli* D88, the 4720 *pol*A$^+$ strain, respectively. The gels of experiments 1 and 3 are shown in Fig. 2. Between 60 and 90% of the input ^{32}P, and 90 and 100% of the input ^3H, were recovered in these bands.

gation, is that the gap in the nascent plus strand should be uniquely located in the R 3 fragment.† In this model the plus strand is synthesized continuously and the minus strand discontinuously in each cycle of RF replication. Although the structure in Fig. 4 contains two continuous parental strands and suggests *de novo* initiation of progeny strands, our results are compatible with the attachment of nascent progeny DNA to parental DNA and the continuous synthesis of plus strands (17).

We have no data that shed light on the significance of the gaps in nascent DNA, but from the results of others (1, 18) it would seem reasonable to hypothesize that the gaps represent regions where an RNA primer had been present in the RF. On this assumption, then, the RNA primer acting to prime synthesis of minus-strand DNA apparently can be synthesized anywhere (or at least at a large number of sites) in the genome, suggesting that there *probably* exists no specific signal for its location. In contrast, as already discussed, there does seem to be site-specific initiation of a round of replication in the region of cistron A and the R3 fragment. Given unidirectional replication, then termination, at least of plus-strand synthesis, must occur here also. Thus, the possible enrichment for ^{32}P as a result of gap-filling in the plus strand in the region of the R3 fragment could reflect some aspect of either initiation (19) or termination of a round of replication.

We thank Dr. Ann Kato for supplying *Neurospora crassa* nuclease and polynucleotide ligase, Katalina Bartok for help with the polyacrylamide gel electrophoresis, and Drs. A. Kornberg and S. Luria for constructive criticism of the manuscript. This research was supported by the Medical Research Council and the National Cancer Institute of Canada.

† This prediction was confirmed as our paper went to press.

1. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 965–969.
2. Sinsheimer, R. L. (1968) *Progr. Nucl. Acid. Res. Mol. Biol.* **8**, 115–169.
3. Iwaya, M., Eisenberg, S., Bartok, K. & Denhardt, D. T. (1973) *J. Virol.* **12**, 808–818.
4. Schekman, R. W., Iwaya, M., Bromstrup, K. & Denhardt, D. T. (1971) *J. Mol. Biol.* **57**, 177–199.
5. Symons, R. H. (1969) *Biochim. Biophys. Acta* **190**, 548–550.
6. Goulian, M., Lucas, Z. J. & Kornberg, A. (1968) *J. Biol. Chem.* **243**, 627–638.
7. Weiss, B. (1971) *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 21, Part D, pp. 319–326.
8. Smith, H. O. & Wilcox, K. W. (1970) *J. Mol. Biol.* **51**, 379–391.
9. Denhardt, D. T. (1969) *J. Biol. Chem.* **244**, 2710–2715.
10. Manor, H., Deutscher, M. P. & Littauer, U. Z. (1971) *J. Mol. Biol.* **61**, 503–524.
11. Edgell, M. H., Hutchison, C. A., III & Sclair, M. (1972) *J. Virol.* **9**, 574–582.
12. Sebring, E. D., Kelly, T. J., Jr., Thoren, M. M. & Salzman, N. P. (1971) *J. Virol.* **8**, 478–490.
13. Rabin, E. Z., Tenenhouse, H. & Fraser, M. J. (1971) *Biochim. Biophys. Acta* **259**, 50–68.
14. Chen, C.-Y., Hutchison, C. A., III & Edgell, M. H. (1973) *Nature New Biol.* **243**, 233–236.
15. Baas, P. D. & Jansz, H. S. (1972) *J. Mol. Biol.* **63**, 569–576.
16. Johnson, P. H. & Sinsheimer, R. L. (1973) *Fed. Proc. Abstracts* **32**, 491 Abstr.
17. Gilbert, W. & Dressler, D. H. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 473–484.
18. Sugino, A., Hirose, S. & Okazaki, R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1863–1867.
19. Denhardt, D. T. (1972) *J. Theor. Biol.* **34**, 487–508.
20. Robberson, D. L., Kasamatsu, H. & Vinograd, J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 737–741.