THE MECHANISM OF REPLICATION OF ϕX174 SINGLE-STRANDED DNA
X. DISTRIBUTION OF THE GAPS IN NASCENT ϕX174 REPLICATIVE FORM DNA.

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SUMMARY

Gaps present in the viral strands of ϕX174 RF (replicative form) DNA extracted from Escherichia coli polA1 cells at the time of RF replication are restricted to a specific location, the region of gene A on the ϕX genetic map. Gaps associated with the complementary strand are found at many locations in the ϕX genome. This was determined by annealing-competition analysis of individual ϕX174 RF DNA fragments produced by Hind restriction endonuclease treatment of ϕX RF DNA specifically labelled in the gap region using α[^{32}P]dCTP and T4 DNA polymerase.

The replication of the single-stranded circular DNA of bacteriophage ϕX174 proceeds through a double-stranded replicative form (RF) intermediate. First, a complementary strand is synthesized on the viral template (1). Then the double-stranded parental RF replicates semiconservatively, as a result of which 10-50 progeny RF molecules are formed in the cell (1). Finally, some of the RF molecules in the cell participate in single-stranded DNA synthesis to yield single-stranded circular molecules which are immediately encapsulated into phage particles (2,3).

Two distinct RF structures are relatively easily identified under most conditions of infection - RF I, which is a covalently closed circular duplex molecule and contains superhelical twists when isolated from the E. coli cell; and RF II, which is a circular molecule with at least one discontinuity in one of the strands (4). The discontinuities in the nascent RF II molecules produced at each of the three stages of the ϕX174 life cycle have been shown to be gaps (5). An analysis of the location of the gaps in the ϕX genome in RF II isolated from E. coli polA1 and polA1^{+} cells during RF replication has been reported previously (6). On the basis of these results we suggested the existence of multiple initiation sites for the synthesis of the complementary strand and a single, unique initiation site for the synthesis of the viral strand (6). Here we report the strand distribution of the gaps in the fragments produced by cleaving the "filled in" RF II molecules with Hind restriction endonuclease.

MATERIALS AND METHODS

Organisms: E. coli HF4720 and the φX174 am3 lysis-defective mutant have been described (5).

Reagents and Enzymes: [methyl-^{3}H]Thymidine (40-60 Ci/mmole) was purchased from New England Nuclear Corp. α[^{32}P]dCTP was prepared essentially by the method of Symons (7). T4 DNA polymerase and T4 polynucleotide ligase were purified through the hydroxyapatite stage (8) and phosphocellulose stage (9) respectively. Endonuclease Hind was purified according to Smith and Wilcox (10).

Preparation of RF II DNA: An E. coli HF4720 polA1 culture in two liters of mT3XD (5) was grown at 37°C with aeration to a concentration of about 4-5 x 10^8 cells/ml and infected with φX am3 phage at an input multiplicity of 5. The culture was labelled with [^{3}H]thymidine, 1 μCi/ml, from 6 to 9 min after infection. Isotope incorporation was terminated by the addition of 1200 ml of a solution containing: 75% ethanol; 20 mM sodium acetate pH 5.5; 4 mM EDTA; 2% phenol (11). The extraction and purification of the φX174 RF II DNA has been described (6).

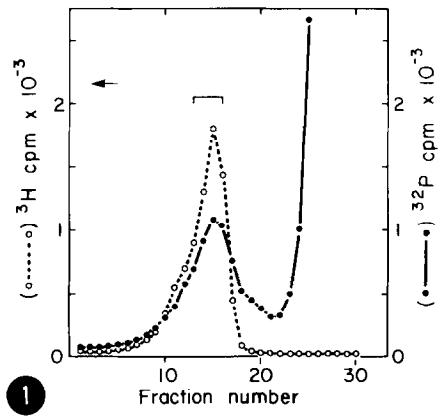
Enzyme Reaction: The "gap-filling" reaction was performed in a total volume of 100 μl containing 10-20 μg of DNA, 6.6 mM $MgCl_2$, 6.6 mM $(NH_4)_2SO_4$, 20 μM each of dATP, dGTP, dTTP and α[^{32}P]dCTP (specific activity of 10 mCi/μmole), 10 mM dithiothreitol, 1 mM ATP, 0.05 M TrisHCl, pH 8.1, 0.02 units of T4 DNA polymerase and 0.6 units of T4 polynucleotide ligase. The reaction mixture was incubated at 37°C for 90 min; the reaction was stopped by adding EDTA to a final concentration of 70 mM.

Preparative Gel Electrophoresis: An apparatus slightly modified from that described by Suendsen (12) was used. A 3.3% acrylamide-0.5% agarose gel in a 0.6 x 8 cm glass tube was prepared as described by Edgell et al. (13). The DNA was electrophoresed for 20 h at room temperature with a current of 3 mA. Every 13 min 0.7 ml fractions were collected. The flow rate was regulated with a Gilson polystaltic pump.

RESULTS

[^{3}H]TdR pulse-labelled φX RF II DNA extracted from E. coli polA1 cells at 9 min after infection was specifically labelled in vitro in the gap region using α[^{32}P]dCTP, T4 DNA polymerase and T4 ligase as described in Materials and Methods. The reacted DNA was purified from the reaction mixture on a neutral sucrose gradient like that shown in Fig. 1. The four peak fractions were pooled and the DNA concentrated by precipitation with 0.1 vol of 3 M sodium acetate, pH 5.5, and 2 vol of 2-propanol (5).

The [^{3}H,^{32}P]RF DNA was then treated with Hind restriction endonuclease, which cleaves φX174 RF DNA into 13 distinct fragments, and the fragments



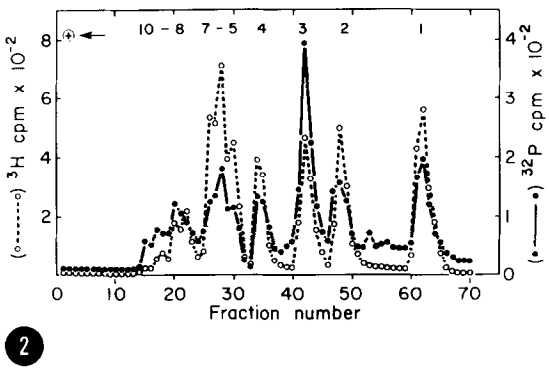


FIGURE 1: Preparative neutral sucrose gradient of [^{3}H]RF II DNA reacted with α[^{32}P]dCTP, T4 DNA polymerase and T4 polynucleotide ligase.

The gaps of RF II DNA were labelled with α[^{32}P]dCTP as described in Materials and Methods. The entire reaction mixture was centrifuged on a linear 5-20% neutral sucrose gradient containing 1 M NaCl, 2 mM EDTA, 0.05 M TrisHCl, pH 8.1 and sucrose. Centrifugation was performed in a SW 50.1 rotor for 3 h at 50,000 rpm and 15°C in a L2 65-B centrifuge. Fractions of 0.15 ml were collected from the bottom of the tube and 2 µl aliquots of each fraction were spotted onto 1 inch squares of Whatman No. 5 filter paper. The papers were dried, 10 ml of toluene-Omnifluor scintillation fluid added and the radioactivity determined in an Intertechnique scintillation counter. ^{3}H, o-----o; ^{32}P, •——•. Sedimentation is from right to left.

FIGURE 2: Preparative agarose-polyacrylamide gel electrophoresis of [^{3}H,^{32}P]RF.

The [^{3}H]RF II DNA, labelled in a "gap-filling" reaction with [^{32}P]dCMP, was purified as described in Fig. 1, and treated with *Hind* restriction endonuclease essentially as described by Edgell *et al.* (13). The reaction was continued for 12 h at 37°C. The reaction mixture was electrophoresed on an agarose-polyacrylamide gel as described in Materials and Methods. 4% of each fraction was taken onto 1 inch square Whatman No. 5 paper and counted as described in the legend to Fig. 1; the remainder was used for the competition-annealing experiments reported in Table 1. ^{3}H, o----o; ^{32}P, •——•. Migration is from right to left. The peaks are designated R1 through R10 starting from the right.

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separated by preparative electrophoresis as illustrated in Fig. 2. The amount of ^{3}H label in the different fragments is proportional to the size of the fragment. The amount of ^{32}P label, which is incorporated *in vitro* into the gaps, appears also to be proportional to the size of the fragment, except for the increased amount of ^{32}P in the R3 fragment.

Fragments R1, R2, R3 and R4 were well separated from each other. Fragments R5, R6, R7, which *in toto* include 6 distinct fragments (13), were not separated under these conditions of electrophoresis. Also, the smaller fragments, R8, R9, and R10 could not be obtained in a pure form. Fractions corresponding to fragments R1, R2, R3 and R4 were pooled separately, and fractions corresponding

TABLE 1: Competition-annealing of ^{3}H and ^{32}P labelled fragments produced from [^{3}H,^{32}P]RF by endonuclease Hind.

Hind Fragment	No competition				Competition			
	^{3}H cpm		^{32}P cpm		^{3}H cpm		^{32}P cpm	
	1	2	1	2	1	2	1	2
R1	12970;	5700	420;	560	7160; (45%)	3500 (39%)	430; (0%)	540 (4%)
R2	10150;	2900	370;	360	4800; (53%)	1600 (45%)	360; (4%)	350 (1%)
R3	12000;	4760	990;	990	7140; (41%)	2700 (42%)	330; (66%)	440 (55%)
R4	10240;	4000	270;	300	5800; (44%)	2200 (45%)	300; (0%)	340 (0%)
R5+R6+R7	32800;	18340	600;	900	21900; (35%)	13800 (25%)	640; (0%)	920 (0%)

(^{3}H,^{32}P)-labelled ϕX174 RF fragments, obtained as described in the legend to Fig. 2, were combined with enough unlabelled RF to give a concentration of 8-10 µg/ml of DNA in 0.25 ml of 10 mM NaCl, 10 mM TrisHCl, pH 7.6, and 0.2 mM EDTA. The DNA was sonicated by an Artec Sonic Dismembrator, using the microprobe set at 50 for 60 sec. Each sample was divided into four aliquots. To two of the aliquots a 20-30 fold molar excess of sonicated unlabelled viral strands was added and the volume of all the samples was adjusted to 100 µl containing (final concentrations) 0.1 M NaCl, 0.1 M TrisHCl, pH 7.6, 0.1mM EDTA in a sealed tube. The DNA was denatured by heating at 100°C for 5 min and then incubated at 60°C to a Cot value of 3-5. The amount of reannealed DNA was determined by precipitation with trichloroacetic acid of the double-stranded DNA remaining after an extensive digestion with N. crassa single-strand specific nuclease. The nuclease reaction and precipitation with trichloroacetic acid were performed as previously described (6). The data presented in the Table are results of analyses of two independent DNA preparations. The % of ^{3}H and ^{32}P label that was competed out by the unlabelled plus strands is given in parentheses. Data of two experiments with two different DNA preparations are presented, each result is the average of duplicate samples. The statistical error in the cpm is 5% or less.

to fragments R5, R6, R7 were combined; the DNA was then precipitated by 2-propanol. The purity of the larger fragments was tested by electrophoresis of a small portion on an analytical agarose-polyacrylamide gel. One band was always obtained with the ^{3}H and ^{32}P label comigrating.

The distribution of the gaps between the two strands of the [^{3}H,^{32}P]RF fragments produced by the Hind endonuclease was determined by means of the annealing-competition experiment described in Table 1. The results from

analyses of two independent DNA preparations, summarized in Table 1, showed that about 40-50% of the ^{3}H label of the different fragments was displaced by the unlabeled viral strand and became sensitive to the N. crassa single-strand specific nuclease. A slightly smaller amount of the ^{3}H label was displaced from the shorter fragments R5+R6+R7. The ^{32}P label, however, could be displaced from only the R3 fragment; 55-65% of the total ^{32}P counts became acid-soluble after treatment with the N. crassa nuclease. The R3 fragment has been mapped in the region of the gene A on the ϕX174 genetic map (13). Essentially all of the ^{32}P in the viral strand of the RF was accounted for by the label displaced from the R3 fragment.

DISCUSSION

As a result of RF replication progeny RF II DNA is formed containing gaps in viral and complementary strands (6). Here, we have demonstrated that the gap(s) in the viral strand is (are) limited to the region of gene A on the ϕX174 genetic map. The multiple gaps in the complementary strand are distributed throughout the ϕX174 genome. Johnson and Sinsheimer (14) also found a gap in the region of gene A in the viral strand of the RF II isolated at late times after infection, at the time of single-stranded DNA synthesis. We do not know yet whether the gaps in the viral strands of the RF II DNA formed as a result of RF replication and single-stranded DNA synthesis have a common nucleotide sequence.

It has been postulated that the gaps in ϕX174 RF II DNA are initiation sites for DNA synthesis (15). The fact that the gaps are characteristically found in nascent RF (5) and the asymmetry in their distribution on the ϕX174 genome strongly support this hypothesis. By analogy to the in vitro situation, where synthesis of an RNA primer for initiation of synthesis of the complementary strand has been demonstrated (16), it seems reasonable to postulate that the gaps in the complementary strand are the sites where RNA primers are synthesized and excised after fulfilling their priming function. The existence of RF molecules with multiple gaps in one strand (6) and the fact that the only gaps which are not restricted to a specific region on the ϕX genome are the gaps in the complementary strand imply discontinuous synthesis of the complementary strand. Although our earlier observations intimated a random distribution of gaps in the complementary strand, recent studies of the average size and base composition indicate a certain specificity in the location of these gaps (to be submitted for publication).

We have suggested (6) that there is de novo initiation of plus strand synthesis in the region of gene A, the synthesis proceeding continuously and clockwise on the genetic map, the gap being the initiation site for the synthesis

of that strand. This is consistent with the origin and direction of RF replication proposed by Baas and Jansz (17). However, inasmuch as the mechanism of termination of a round of replication is not understood, we cannot exclude the possibility that the formation of a gap in the plus strand reflects the mechanism of separation of the two template strands at the termination of a round of replication.

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