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Discontinuous replication of replicative form DNA from bacteriophage φX174

(nascent short pieces of both strands/RNA-linked DNA pieces/rolling circle mechanism)

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ABSTRACT **Bacteriophage φX174 DNA has been labeled with short pulses of [^{3}H]thymidine during synthesis of replicative form molecules in infected *Escherichia coli* HF4704 cells. The replicating φX174 DNA was isolated and analyzed by sedimentation in an alkaline sucrose gradient. During a brief pulse (5 sec at 30°), the radioactivity incorporated into the complementary strand was found in chains much shorter than one genome length. Of the radioactivity incorporated into the viral strand, two-thirds was in the short pieces and the rest was in chains of one genome length or longer. RNA attachment to the 5′ end of both strand components of the nascent short pieces was shown by the appearance of spleen exonuclease-digestable nascent molecules after alkali treatment. These observations suggest that the viral as well as the complementary strand is synthesized by the discontinuous mechanism with RNA primers during replication of duplex φX174 DNA.**

The chromosome of bacteriophage φX174 is among the smallest known. It is composed of single-stranded circular DNA of about 5400 nucleotides, which is only several times longer than a short nascent DNA found in discontinuous replication of many prokaryotic organisms (1). The phage DNA is replicated in three distinct stages: (*i*) conversion of single-stranded viral DNA to duplex replicative form (RF), (*ii*) RF replication, during which multiple copies of RF are synthesized semiconservatively, and (*iii*) synthesis of the viral DNA with the complementary strand (C strand) of RF used as template (2).

The rolling circle model has been proposed for the replication in stages *ii* and *iii* (3–5). According to this model for stage *ii* replication, the viral strand (V strand) is synthesized continuously in the overall 5′ → 3′ direction, producing a chain of longer than unit length, and the C strand is synthesized discontinuously in the overall 3′ → 5′ direction, using the displaced free V strand as the template (4). The presence of only C strand short pieces during RF replication has been reported (6, 7).

The purpose of this report is to describe the mechanism of replication of φX174 RF DNA. We have found that after very short pulses most of the radioactivity is recovered in nascent short pieces of the V and C strands. Furthermore, the labeled short pieces of both strands are linked to RNAs which probably have primed the synthesis of these short pieces. These results suggest the discontinuous synthesis of the V and C strands.

MATERIALS AND METHODS

Organisms and Chemicals. *Escherichia coli* HF4704 is *uvrA*$^-$ and requires thymine at 37° but not at 30° (8, 9). φX174 *am*3 is a lysis-defective cistron *E* mutant. [*methyl*-^{3}H]Thymidine (43.1 or 50.8 Ci/mmol) was purchased from New England Nuclear Corp. Lysozyme and RNase IA were obtained from Worthington Biochemical Corp. RNase T1 was provided by T. Uchida. These RNases were used after heating at 90° for 10 min in 10 mM sodium acetate buffer (pH 5.0). *E. coli* tRNA was a gift of S. Nishimura.

Infection Procedure and Pulse Labeling of φX174-Infected *E. coli*. *E. coli* HF4704 cells were grown in Tris/phosphate/glucose/casein amino acids (TPG-CAA) medium (10) plus thymine (2 μg/ml) at 37° to 4×10^8 cells per ml. The cells were pelleted by centrifugation, washed once with starvation buffer (11), and resuspended in the same buffer at 8×10^8 cells per ml. In order to synchronize replication cycles of φX174 DNA, the cells were then starved at 30° for 90 min, and phages were added at a multiplicity of infection of 10. After 5 min at 30°, an equal volume of 2-fold-concentrated TPG-CAA medium was added and the culture was incubated at 30°. [^{3}H]-Thymidine was added to a concentration of 0.1 μM, 16 min after infection during the period of RF replication. After various periods of labeling, an equal volume of an ethanol/phenol mixture (12) was poured into the culture.

Isolation of φX174 RF Molecules. φX174 RF molecules were separated from bacterial DNA by neutral sucrose gradient sedimentation after gentle lysis of the infected cells as described by Ray and Schekman (13) except that the lysozyme treatment was performed at 0° for 30 min.

Alkaline Sucrose Gradient Sedimentation. The fractions from the neutral sucrose gradient containing RF molecules were pooled and diluted with a half volume of water. After the addition of *E. coli* tRNA to a concentration of 50 μg/ml, the sample was concentrated by isopropanol precipitation. The precipitated DNA was resuspended in 0.5% sodium *N*-lauroyl sarcosinate/10 mM EDTA and NaOH was added to a final concentration of 0.2 M. The DNA solution was incubated at 37° for 20 min, chilled, layered on top of an alkaline sucrose gradient (14), and centrifuged in a Beckman SW 27, SW 41 Ti, or SW 56 Ti rotor.

Preparative Separation of the Complementary and the Viral Strands. Unlabeled or ^{3}H-labeled φX174 RFI was prepared from infected HF4704 cells as described by Altman and Denhardt (15), except that digestion with RNase IA and RNase T1 and gel filtration on Sephadex G-200 were performed before ethidium bromide/CsCl equilibrium centrifugation. After boiling of RFI in 0.2 M NaOH for 5 min and chilling, single-stranded molecules were isolated by alkaline sucrose gradient sedimentation. The C and V strands were prepared as described previously (16), except that 30–40 μg of single-stranded DNA was mixed with the same amount of poly(U,G) (pretreated with 0.1 M NaOH at 37° for 27 min) in 10 mM Tris·HCl (pH 7.6)/1 mM EDTA. Hybridization experiments with the unlabeled and labeled strands indicated that the isolated strands were sufficiently pure (Fig. 1).

DNA·DNA Hybridization. DNA·DNA hybridization was

Abbreviations: RF, duplex replicative form (RFI is double-stranded, closed-circular, and superhelical); V strand, viral strand; C strand, complementary strand; SSC, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate); 5′-OH DNA, 5′-hydroxyl-terminated DNA.

* *Deceased.*

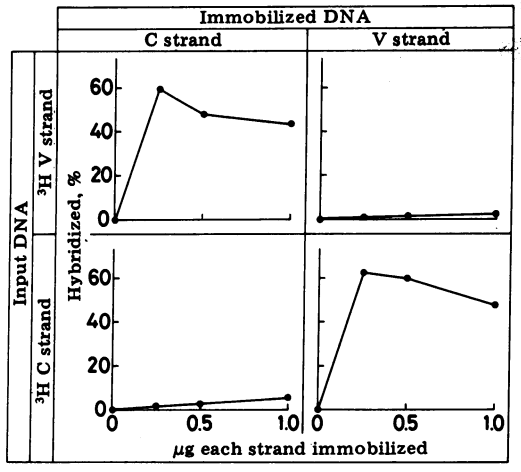

FIG. 1. Model hybridization experiments with labeled and unlabeled DNA strands; 0.02 μg (400 cpm) of each fragmented labeled strand was hybridized with various amounts of the unlabeled strand as indicated. The percentage of labeled DNA bound to the DNA filter is plotted against the amount of immobilized unlabeled DNA.

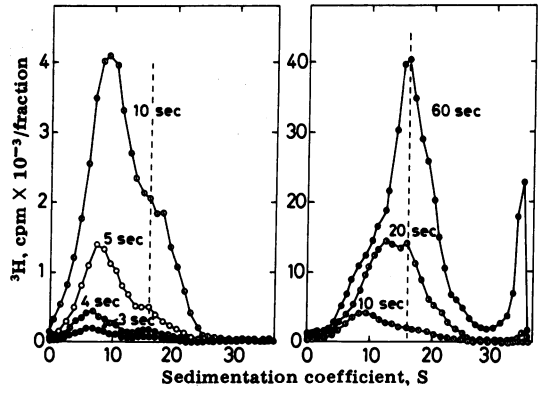

FIG. 2. Alkaline sucrose gradient sedimentation of pulse-labeled RF DNA from φX174-infected *E. coli* HF4704. Cultures (20–50 ml) of φX174-infected *E. coli* HF4704 cells were pulse labeled with 0.1 μM [³H]thymidine (43.1 Ci/mmol) for the indicated times 16 min after infection at 30°, and RF molecules were isolated by neutral sucrose gradient sedimentation as in *Materials and Methods*. After concentration and alkali denaturation, the RF molecules were sedimented, together with φX174 [¹⁴C]DNA [18 S (18)] marker, through an alkaline sucrose gradient. The broken line indicates the position of a genome-length linear φX174 DNA (16 S), calculated from the position of ¹⁴C-labeled marker.

performed as described by Kainuma-Kuroda and Okazaki (16) with 0.25 μg of separated strands immobilized on each membrane filter. To examine the effect of input DNA concentration on the hybridization, each fraction of the pulse-labeled short pieces was tested at various DNA concentrations. The result showed that in each fraction the percentages of the hybridization were virtually constant at input DNA concentrations less than 0.01 μg/ml. The hybridization with the fractions of the nascent short pieces was performed under these conditions.

Preparation of Nascent DNA Pieces for the Spleen Exonuclease Assay of 5′-Hydroxyl-Terminated DNA (5′-OH DNA). A culture (720 ml) of φX174-infected *E. coli* HF4704 cells was pulsed labeled with 0.1 μM [³H]thymidine (43.1 Ci/mmol) at 30° for 5 sec 16 min after infection, and RF molecules were isolated. After dialysis against standard saline citrate (SSC; 0.15 M NaCl/0.015 M sodium citrate) containing 25% sucrose, 10 mM EDTA, and 1% sodium dodecyl sulfate, the sample was treated successively with Pronase (1 mg/ml) and phenol, and then dialyzed against 0.1 × SSC/0.01% sodium dodecyl sulfate. The sample was concentrated to 6 ml with a rotary evaporator and dialyzed against 0.1 × SSC/0.01% sodium dodecyl sulfate/1 mM EDTA. After heat denaturation (100°, 4 min), the nascent pieces (100–800 nucleotides) were isolated by neutral sucrose gradient sedimentation followed by Cs_2SO_4 equilibrium centrifugation and the DNA pieces were phosphorylated with polynucleotide kinase and ATP. The details of the preparation procedure and the assay of 5′-OH DNA by spleen exonuclease have been described (17).

RESULTS

Sedimentation Analysis of the Nascent DNA of Semiconservatively Replicating φX174 RF Molecules. The φX174 RF DNA that had been pulse labeled during semiconservative replication was separated from labeled bacterial DNA by sedimentation through a neutral sucrose gradient. DNA·DNA hybridization analyses showed that roughly 80% of labeled φX174 RF DNA was recovered in the sucrose gradient. More than 95% of the radioactivity in the sucrose gradient was in φX174 RF DNA and annealed with both the V and C strands in approximately equal proportion, and the rest was in *E. coli* DNA. The purified RF DNA was denatured and analyzed by sedimentation through an alkaline sucrose gradient (Fig. 2). After 3- to 5-sec pulses, most of the radioactivity was incorporated into the nascent pieces with an average sedimentation coefficient of 7 S, though some radioactivity was also found in linear molecules of one genome length (16 S) or longer. During pulses up to 20 sec, the average sedimentation coefficient of the short pieces increased gradually. Extending the pulse time to 60 sec resulted in the increase of radioactivity in the 16S component; a component sedimented onto a cushion and probably represents alkali-denatured RFI.

Hybridization of the Pulse-Labeled DNA with the V and C Strands. The distribution of the V and C strands in the pulse-labeled DNA displayed by an alkaline sucrose gradient was analyzed by DNA·DNA annealing (Fig. 3). After a 5-sec pulse, almost all the label incorporated into the C strand was present as short pieces, whereas two-thirds of the label incorporated into the V strand was in short pieces and the rest was in chains of one genome length or longer (Fig. 3*A*). In the 20-sec-labeled C strand DNA (Fig. 3*B*), the relative amount of the radioactivity increased in chains of intermediate size (6–16 S) and of genome-length linear DNA. In the 20-sec labeling of the V strand, much of the radioactivity was in chains of longer than one genome length, although a peak of radioactivity was still observed in short pieces.

Analysis of RNA-Linked Nascent DNA Pieces by the Spleen Exonuclease Method. Five-second-labeled nascent pieces (100–800 nucleotides) were examined for the presence of RNA-linked DNA pieces by the spleen exonuclease method, which measures the proportion of the radioactive 5′-OH DNA produced by alkali or RNase treatment of the nascent pieces (17). As shown in Fig. 4 *B* and *C*, half of the radioactive short pieces became susceptible to spleen exonuclease after these treatments. In the control without alkali or RNase treatment (Fig. 4*A*), no 5′-OH DNA was detected. To explore RNA attachment to nascent pieces of both strands, the alkali-treated nascent pieces were separated into V and C strand components by hybridization and analyzed by the same method (Fig. 5). Alkaline hydrolysis produced the 5′-OH nascent molecules from each strand in similar proportion, indicating RNA attachment to the nascent pieces of both strands. These results suggest that

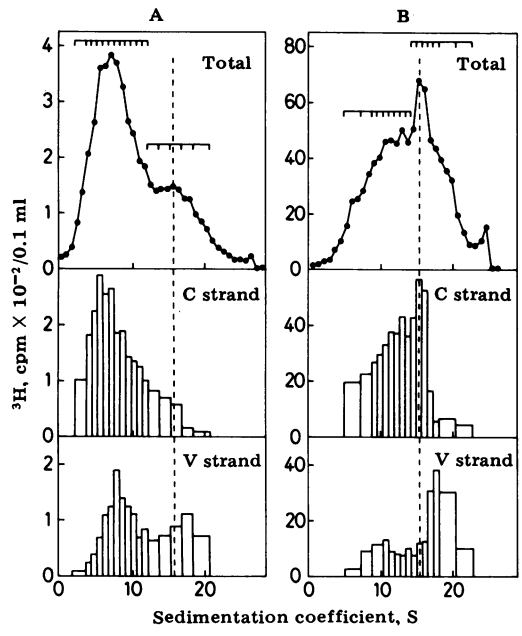

FIG. 3. The size distribution of the C and V strand components of pulse-labeled DNA. Cultures [360 ml (*A*) and 50 ml (*B*)] of φX-174-infected *E. coli* HF4704 cells were pulse labeled with 0.1 μM [^{3}H]thymidine (43.1 Ci/mmol) for 5 sec (*A*) or 20 sec (*B*) at 30°. The labeled RF DNA was sedimented through an alkaline sucrose gradient. φX174 [^{14}C]DNA was centrifuged in the same run but in a separate tube and the position of unit-length linear φX174 DNA (broken line) was calculated. Hybridization tests were performed with the bracketed fractions. In each hybridization test, the input DNA had 80–2200 cpm. The amounts of the radioactive C and V strand components in each fraction were calculated using the following equations:

Radioactivity of the C strand component = total radioactivity of the fraction × $v/[(c/1.3) + v]$; radioactivity of the V strand component = total radioactivity of the fraction × $(c/1.3)/[(c/1.3) + v]$. v and c represent the percentages of the labeled DNA hybridized with the V strand and the C strand, respectively. The factor of 1.3 corrects for the higher thymine content of the V strand.

the synthesis of the nascent pieces of both daughter strands of φX174 RF DNA is primed by RNA.

DISCUSSION

When φX174-infected cells are briefly pulse labeled with [^{3}H]thymidine during the RF replication stage, most of the radioactivity incorporated into φX174 DNA is found in short pieces (Fig. 2). Hybridization experiments have revealed both V and C strand nascent short pieces (Fig. 3). The result of the experiment with spleen exonuclease indicates that RNA is linked to the nascent short pieces of both strands (Fig. 5), suggesting that RNA primes chain initiation for both strands. Because the replication of φX174 RF DNA proceeds unidirectionally (19, 20), these findings suggest that both daughter strands are synthesized discontinuously. Studies carried out by C. Yasumoto and J. Hurwitz (unpublished) suggest that *in vitro* both strands of φX174 RF DNA are synthesized discontinuously.

Though the short pieces of both strands exist, the mode of joining of the V and C strand pieces may be different. In contrast with the radioactivity incorporated into the C strand, which is found almost exclusively in the short pieces after a brief pulse, some proportion of the radioactivity incorporated into the V strand is found in the chains of unit length or longer (Fig. 3). Taking into account the reported overall direction of the replication of each strand (19, 20) and the asymmetric labeling

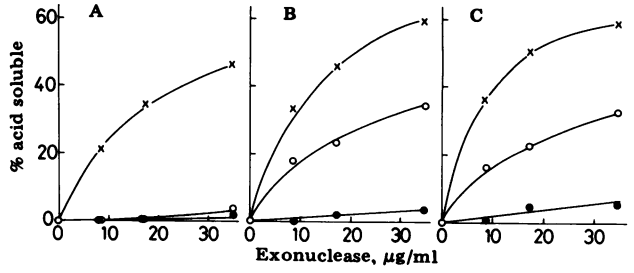

FIG. 4. Spleen exonuclease digestion of alkali- or RNase-treated nascent pieces from φX174-infected *E. coli* HF4704. One-third of the sample after phosphorylation as in *Materials and Methods* was withdrawn and divided further into three portions, *A*, *B*, and *C*, of 5,000–8,000 cpm each; *A* was left untreated; *B* was treated with 0.15 M NaOH at 37° for 20 hr; *C* was treated with 100 μg of RNase IA per ml and 10 μg of RNase T1 per ml in 10 mM Tris·HCl (pH 8.0)/0.1 mM EDTA at 37° for 90 min. Each portion was digested with spleen exonuclease directly (○), after polynucleotide kinase treatment (●), or after alkaline phosphatase treatment (×), as described previously (17).

of the two strands in RFI after a short pulse with [^{3}H]thymidine (4), the results obtained in the present study can be interpreted in terms of a modified rolling circle model illustrated in Fig. 6. The extruded V strand serves as a template for the elongation of a new C strand in the overall 3′ → 5′ direction. The short pieces are then joined to each other. The elongation of the V strand in the overall 5′ → 3′ direction also proceeds discontinuously using the circular C strand as a template, but its nascent pieces could be joined directly to the older part of the strand of one genome length or longer while it is still being synthesized. Therefore, short and long growing chains of the V strand can exist at a given moment and be labeled by a brief pulse. The present results, however, do not rule out the alternative possibility that some portion of the V strand might be synthesized continuously as long stretches. In the rolling circle type replication of P2 phage DNA, similar asymmetric incorporation of the radioactivity between two strands has been observed (12)

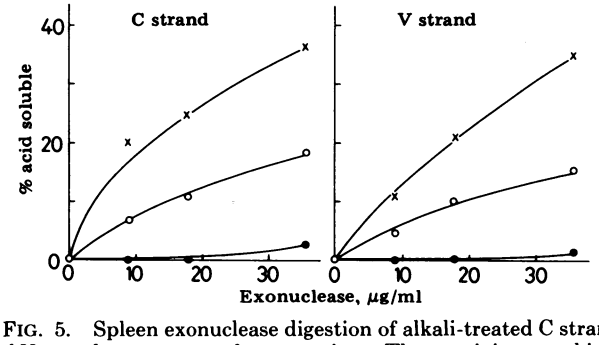

FIG. 5. Spleen exonuclease digestion of alkali-treated C strand and V strand components of nascent pieces. The remaining two-thirds of the sample of Fig. 4, after phosphorylation, was treated with 0.15 M NaOH at 37° for 20 hr and split into three portions, A, B, and C, with 12,000 cpm each. Each was hybridized with the V and C strands immobilized on membrane filters directly (A), after polynucleotide kinase treatment (B), or after alkaline phosphatase treatment (C). In each portion, the hybridization was performed in 10 vials each containing input DNA of 1200 cpm and the two membrane filters, one immobilized with the V strand and the other with the C strand. Percentages of hybridization with the V and C strands were about 28% and 22%, respectively. After hybridization, the membrane filters were washed in 3 mM Tris base and labeled short pieces annealed to each strand were eluted by dipping each type of membrane filter in 2.5 ml of 0.1 M NaOH/10 mM EDTA at room temperature for 10 min. After dialysis against 10 mM Tris·HCl (pH 8.0)/0.1 mM EDTA and concentration, each strand component of A (○), B (●), and C (×) was digested with spleen exonuclease as in Fig. 4.

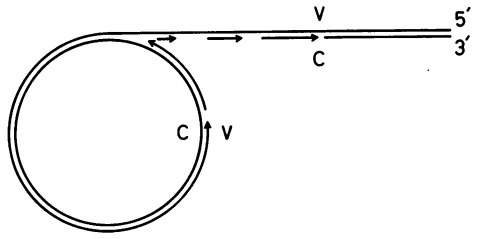

FIG. 6. Proposed structure of replicating double-stranded DNA of ϕX174.

and RNA-linked nascent DNA molecules of both strand components have been detected (21).

During the present study, it was noticed that the conditions of ϕX174 development or of host cell culture influence the extent of the discontinuity of both strands of ϕX174 nascent DNA. In ϕX174-infected *E. coli* HF4704, the discontinuity is more prominent than in ϕX174-infected *E. coli* C strain (compare Fig. 2 in this paper with figure 17 of ref. 21). *E. coli* HF4704 cells were routinely grown at 37° prior to the ϕX174 infection and development at 30°. With bacteria grown at 30°, the discontinuity was less pronounced, although the nascent pieces of both strands were also clearly detectable under this condition (unpublished observation). Many factors may possibly influence the discontinuity of nascent DNA. Frequency of chain initiation reaction relative to chain elongation rate might have an important influence on the stretch of short piece synthesis, especially on that of the V strand. The excision-repair system of misincorporated uracil residues in DNA (22) might also influence the extent of discontinuous synthesis of nascent DNA in response to metabolic changes of precursor nucleotides in cells. If the excision-repair system operates, the products will be RNA-free DNA fragments. The present results, however, indicate that after a very short pulse (5 sec at 30°), half of the nascent short pieces of both strands are linked to RNA and the rest are RNA-free DNA. Therefore, the synthesis of at least half of the short pieces may be initiated by RNA primers. The RNA-free DNA may contain molecules formed after the removal of RNA from the nascent short pieces; the proportion of these molecules is unknown.

The presence of only the C strand short pieces during RF replication has been reported (6, 7). These analyses might have been performed under conditions extremely favorable for long-stretch synthesis. Alternatively, the rapid joining of the V strand short pieces might have caused the apparent discrepancy between these reports and ours.

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