

*THE PROCESS OF INFECTION WITH BACTERIOPHAGE ϕ X174,
XXII. SYNTHESIS OF PROGENY SINGLE-STRANDED DNA**

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In a previous article,¹ it was demonstrated that of the labeled thymidine incorporated into the replicative form (RF) of ϕ X-DNA during the period of RF replication (3–12 or 15 min after infection), approximately half was transferred to virus during the subsequent period of single-strand DNA synthesis, while half remained in RF molecules. In this paper experiments are presented which permit a definition of the label remaining in the RF and which clarify the role of the RF in the synthesis of the progeny single-stranded DNA.

Experimental.—Most of the relevant techniques were described in our preceding article.¹

Exonuclease III digestion: Digestions with exonuclease III² were performed in 0.05 M tris buffer, pH 8.0, in the presence of 5 mM MgCl₂ and 2 mM mercaptoethanol for 4 hr at 37°C. Approximately 800 units of exonuclease III were added for 50 μ g DNA in a total volume of 1.4 ml.

DNA-DNA hybridization: The annealing technique of Denhardt³ was used. Solutions of labeled DNA (containing about 1 μ g DNA) were annealed with treated nitrocellulose filters (HAWP, Millipore) previously loaded with 100 μ g unlabeled viral ϕ X-DNA, washed, and counted.

Results.—*The nature of the persistent RF label:* If RF is labeled with H³-thymine during the period of RF replication and the label is subsequently chased, approximately one half of the label remains in the RF.¹ When cells were infected with ϕ Xam3 at 30°, labeled with H³-thymine during the period 5–18 minutes after infection, and the label subsequently chased with cold thymine for 60 minutes, nearly one half the label remained in RF, distributed as 4 RFII:1 RFI. When this RFII was centrifuged to equilibrium in alkaline CsCl (Fig. 1), all of the label was found at the buoyant density of the complementary strand. When this RFII was analyzed by zone sedimentation through a preformed alkaline CsCl gradient (Fig. 2), approximately 80 per cent of the label sedimented at the rate of the ring form of ϕ X-DNA, while 20 per cent sedimented at the rate of the linear form.

Evidently, in this RFII, which contained the bulk of the label remaining after the chase, the label was exclusively in the complementary strand, very largely in the form of a closed ring.

In a similar experiment, in which cells were labeled with H³-thymine during the period 0–14 minutes after infection, at 37°C, and the label was then chased with cold thymine for 20 minutes, again about one half the label remained in RF (6 RFII:1 RFI). When this RFII was analyzed by zone sedimentation through a preformed alkaline CsCl gradient, the persistent label was found (Fig. 3a) partly in circular DNA, partly in whole linear DNA, and partly in DNA fragments, presumably from RFII molecules with more than one cut in a strand. When the circular and whole linear DNA components from the gradient of Figure 3a were separately centrifuged to equilibrium in alkaline CsCl (Figs.

3b, c), the label was found, in both cases, almost exclusively at the buoyant density of complementary DNA.

This experiment confirms the result of that previously described, in that the label persisting in RF is found in the complementary strands, but indicates that there may be some variability in the extent to which these complementary strands remain intact, dependent upon experimental conditions.

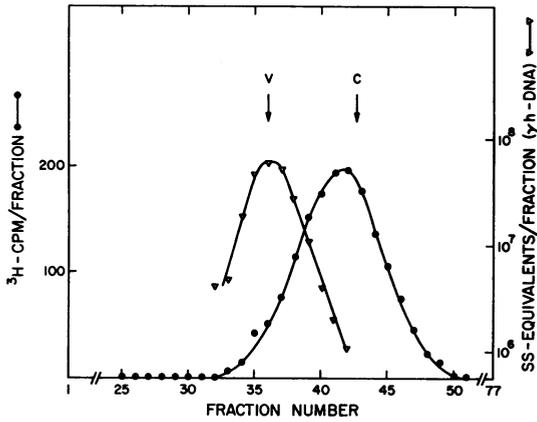


FIG. 1.—Analysis of RFII isolated by a zone sedimentation (through a neutral CsCl gradient) of DNA extracted from cells labeled during the period of RF replication (5–18 min after infection at 30°C) and subsequently “chased” for 60 min. Equilibrium distribution in alkaline CsCl gradient ($\phi X\gamma h$ -DNA added as marker).

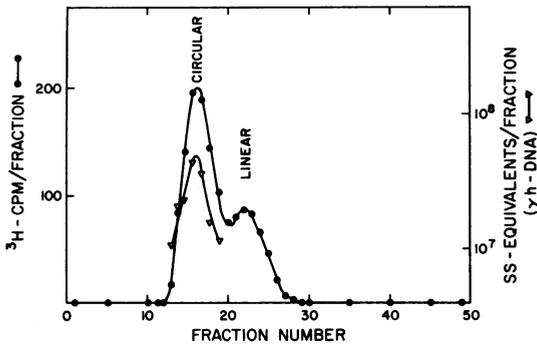


FIG. 2.—Distribution of H^3 -label in RFII of Fig. 1 after sedimentation through a preformed alkaline CsCl gradient ($\phi X\gamma h$ -DNA added as marker).

Label incorporated into “late” RFII: Lindqvist and Sinsheimer⁴ showed that H^3 -thymine incorporated during the period of single-strand progeny DNA synthesis was first detected in RFII, before transition to single-stranded DNA. When such pulse-labeled RFII from cells (labeled with H^3 -thymidine for 1 min at 30°C at 45 min after infection) is analyzed by zone sedimentation through a preformed alkaline CsCl gradient (Fig. 4a), it can be seen that all the label is to be found in a component sedimenting with the velocity of linear ϕX -DNA. When this component is centrifuged to equilibrium in an alkaline CsCl density gradient (Fig. 4b), all the label is found at the buoyant density of viral ϕX -DNA.

Evidently the label incorporated into RFII during the period of progeny single-strand DNA formation is present only in the viral strand, which is open at one link.

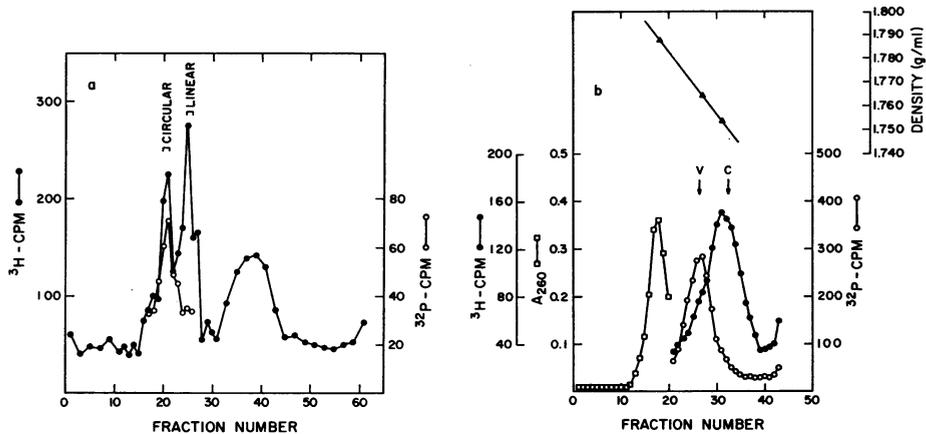


FIG. 3.—Analysis of RFII isolated by zone sedimentation (through a neutral CsCl gradient) of DNA extracted from cells labeled during the period of RF replication (0–14 min after infection at 37°C) and subsequently chased for 20 min.

(a) Distribution of H^3 -label after sedimentation through a preformed alkaline CsCl gradient (P^{32} - ϕX -DNA added as marker).

(b) Equilibrium distribution in an alkaline CsCl density gradient of the "circular" component from Fig. 2a (*M. lysodeikticus* DNA (A_{260}) and P^{32} - ϕX -DNA added as markers).

(c) Equilibrium distribution in an alkaline CsCl density gradient of the "linear" component from Fig. 2a (*M. lysodeikticus* DNA (A_{260}) and P^{32} - ϕX -DNA added as markers).

At the end of a short pulse of H^3 -thymine during this period of single-strand DNA formation, a small fraction of incorporated label is found as RFI.⁴ To examine the distribution of label in this component, such "late" pulse-labeled RFI was isolated from a zone sedimentation through neutral CsCl, treated with sufficient pancreatic deoxyribonuclease to produce an average of one single-strand break per molecule, and the product centrifuged to equilibrium in an alkaline CsCl density gradient. As can be seen in Figure 5, the label is to be found only in denatured, unattached RFI (40%) and in DNA with the buoyant density of viral ϕX -DNA (60%). Evidently only the viral strand is labeled in the pulse-labeled RFI as well as in the RFII.

Digestion with exonuclease III: If the open viral strand of "late" pulse-labeled RFII has a free 3'OH end, it should be susceptible to digestion by exonuclease III. When such an RFII preparation (21,000 cpm/ml or approximately 5 $\mu\text{g}/\text{ml}$) was digested with exonuclease III for four hours at 37°C, the acid-precipitable label decreased by 88 per cent. During this incubation the infectivity⁵ of the solution increased some sevenfold⁶ during the first two hours

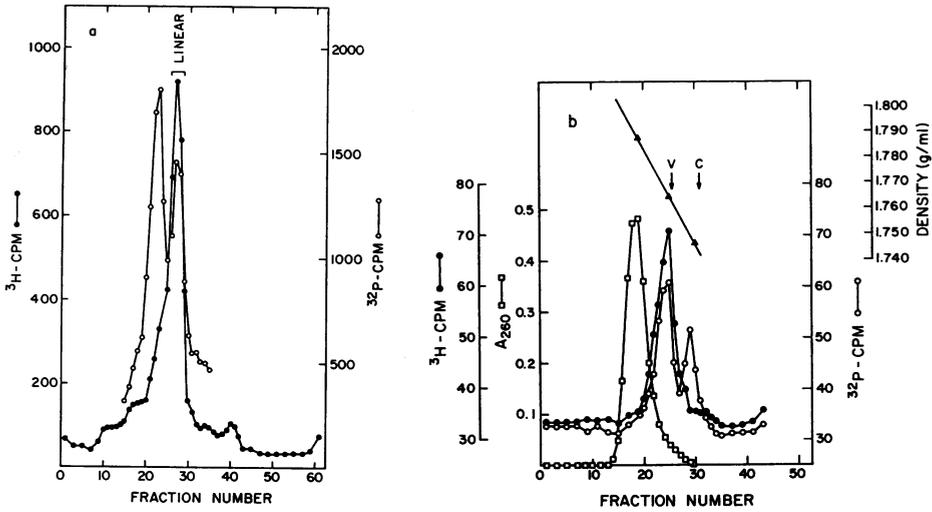


FIG. 4.—Analysis of RFI (isolated by zone sedimentation through a preformed neutral CsCl gradient) labeled in a 1-min pulse at 30°C during the period of progeny single-strand DNA formation (at 45 min after infection).

(a) Distribution of H³-label after zone sedimentation through a preformed alkaline CsCl gradient (P³²-RFII, uniformly labeled, added as a marker).

(b) Equilibrium distribution in alkaline CsCl density gradient of “linear” component from Fig. 4a (*M. lysodeikticus* DNA (A₂₆₀) and P³²-RFII, uniformly labeled and randomly nicked, added as density markers).

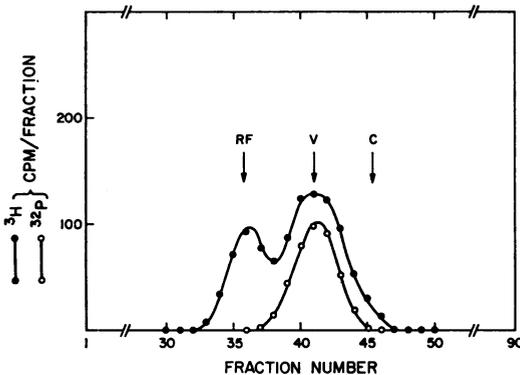


FIG. 5.—Distribution of label in RFI, isolated by zone sedimentation through a preformed neutral CsCl gradient of the DNA extracted after a 1-min pulse of H³-thymine during the period of progeny single-strand DNA formation. This RFI was briefly treated with DNase and the product centrifuged to equilibrium in an alkaline CsCl density gradient (P³²- ϕ X-DNA added as a marker).

(as a consequence of the release of the more highly infective, single complementary strands) and then remained constant, indicating that the enzyme preparation contained no significant endonuclease impurities.

These experiments demonstrate that the open viral strand of the late pulse-labeled RFI has a free 3'OH end. They also indicate that in the bulk of the pulse-labeled RFI, the complementary strands must be closed (Fig. 2) or else the digestion with exonuclease III could not have proceeded essentially to completion.

Hybridization experiments: Although the alkaline equilibrium density

gradients of Figures 4b and 5 indicate that most of the label incorporated into RF during the period of single-stranded DNA formation goes into the viral DNA strands, a small incorporation into complementary strands could not be detected by these methods. To test this possibility, pulse-labeled late RFII (labeled for 1 min at 30°C at 45 min after infection) was denatured and annealed with a nitrocellulose filter carrying 100 μ g of unlabeled viral DNA. Control experiments were performed with labeled viral DNA and with denatured "artificial" RFII, isolated after deoxyribonuclease digestion of labeled "early" RFI, of equal specific labeling in both strands. As can be seen in Table 1,

TABLE 1. DNA-viral DNA hybridization.

Test DNA	Cpm added	Cpm annealed*	Per cent annealed	Per cent complementary strands
ϕ X-DNA	320,000	≤ 50	≤ 0.02	0
"Artificial" RFII†	112,000	2570	2.3‡	50
"Late" pulse-labeled RFII	65,000	122-222	0.19-0.34	4.6-8.5

* Background has been subtracted.

† "Artificial" RFII is prepared by light DNase digestion of uniformly labeled "early" RFI.

‡ In a uniformly labeled RF, 2.3% of the label corresponds to 5.4% of the complementary strands.

between 0.19 per cent and 0.36 per cent of the label in late RFII anneals to the filter, as compared to 2.3 per cent of the label of the "artificial" RFII and less than 0.02 per cent of the viral DNA. From this data and allowing for the difference in thymine contents of the viral and complementary strands, it may be calculated that between 4.6 per cent and 8.5 per cent of the strands labeled during a late pulse are complementary strands.

Discussion.—From the results presented in this and previous papers,^{1, 4, 7} three phases of DNA replication may be discerned during infection with bacteriophage ϕ X174.

In phase I the single-stranded DNA ring is converted to a double-stranded DNA ring (RF), which may be open or closed (RFII or I). This process is carried out by pre-existing host-cell enzymes⁷ and has been duplicated *in vitro*.⁸

In phase II, semiconservative replication of the double-stranded DNA ring involving, frequently, only the RF at a bacterial "site"⁹ is observed. This process requires the participation of viral-specified function.^{7, 10} In this replication only the RFII form is active;¹¹ the parental DNA strand remains at the "site," and the nascent RF appears as RFII⁴ of two types.¹

In phase III, the viral strands of the progeny RF are repeatedly displaced into virus particles with the concomitant synthesis of new viral strands upon the persistent complementary strands. In the RFII pulse-labeled during this process the viral strands are open, the complementary strands in greater part are closed. Evidently the viral strands must be closed at some stage before completion of the virus particles. During this phase a low level of continuing synthesis of complementary strands can be detected, possibly a consequence of a small, continued semiconservative RF replication at the "site."

Summary.—That label which, after incorporation into RF during the period of RF replication, persists in RF after a period of progeny single-strand DNA

formation is only in the complementary strands of RF. These strands are, under some circumstances, largely closed.

Label incorporated into RFII (or I) during pulses in the "late" period of single-stranded DNA synthesis is almost exclusively in viral strands, which are open. These strands in RFII have a free 3'OH end and the complementary strands of such RFII molecules are for the most part closed.

A low rate of synthesis of complementary strands continues during the period of progeny single-strand DNA formation.

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