

Stages in the Replication of Bacteriophage ϕ X174 DNA in vivo

ROBERT L. SINSHEIMER, ROLF KNIPPERS, AND TOHRU KOMANO
Biology Division, California Institute of Technology, Pasadena, California

Research with bacteriophage ϕ X174 over the past few years has led to the recognition of three distinct stages in the replication of the viral DNA. An outline of the replicative process is formulated in Fig. 1 (Sinsheimer et al., 1962; Lindqvist and Sinsheimer, 1968; Knippers et al., 1968; Komano et al., 1968).

Upon entry into the cell, the single-stranded DNA of the virus, which is a ring DNA, is quickly converted to a double-stranded DNA ring, the replicative form or RF. Two centrifugal forms of the double-stranded ring are found in the cell: the supercoiled form with both strands covalently closed (RFI), and the open form with one strand open (RFII).

The conversion from single- to double-stranded ring is performed by pre-existent host enzymes. No viral functions are needed for this step. It can occur in the presence of high concentrations of chloramphenicol, or in amino acid auxotrophs starved for the essential amino acid. This step has been closely duplicated in vitro with the aid of the

host DNA polymerase and the host polynucleotide ligase (Goulian et al. 1967).

After formation, the RF becomes associated with a pre-existent essential site within the bacterial cell. The number of such sites is limited and depends upon the physiology of the cell. In previously starved cells it is usually one (Yarus and Sinsheimer, 1967). Unless an RF becomes associated with such a site it cannot replicate or give rise to progeny.

After association with such a site the RF replicates semiconservatively (Denhardt and Sinsheimer, 1965). This replication is such that the input viral DNA strand always remains associated with the site, exchanging partners at each replication. This site sediments with the membrane fraction of the cell lysate (Knippers and Sinsheimer, 1968).

This replication of the RF requires both host and viral function (Tessman, 1966; Lindqvist and Sinsheimer, 1967). It is blocked in certain host mutants, and by prior ultraviolet irradiation of the

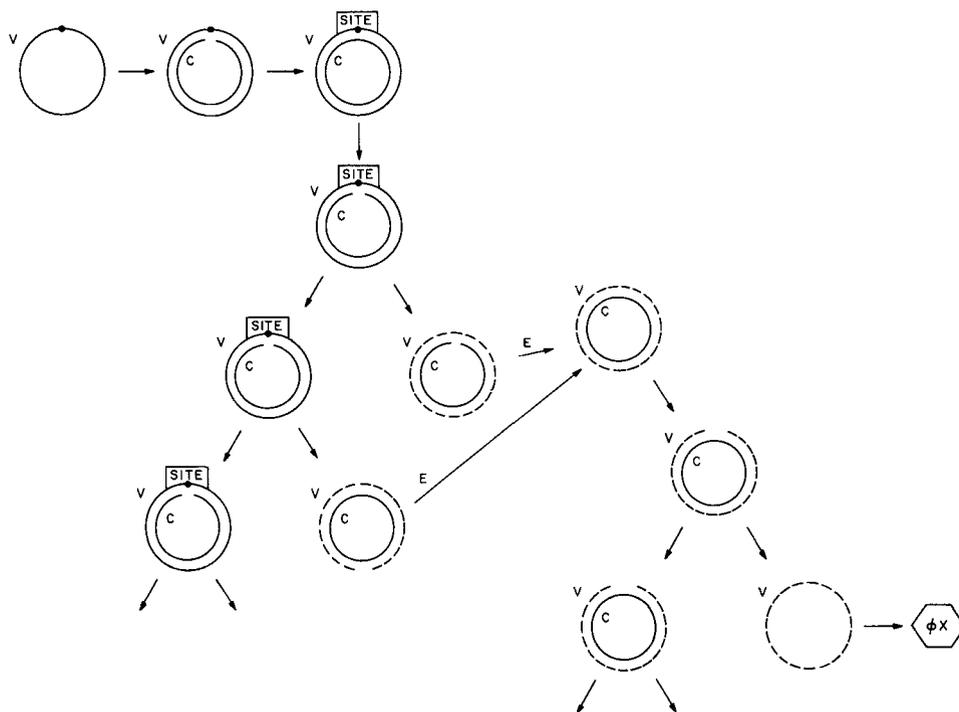


FIGURE 1. Schematic outline of the replication cycle of bacteriophage ϕ X174.

host. The product of viral cistron VI is needed for RF replication (Sinsheimer, 1968).

Pulse-labeling experiments indicate that the RF which is replicating on the site is in the open form, with one strand open. In virtually all of such open RF molecules the viral strand is closed while the complementary strand is open.

Similarly the nascent daughter RF molecules released from the site and sedimenting free of the membrane fraction are initially in the open form. Again, in approximately half of these the viral strand is open; in the other half the complementary strand. The significance of this distribution is unclear. Conceivably it represents an alternation of open strands in the replicative cycle.

RF replication is initiated about 2-3 min after infection (at 37°C) and continues until about 12 min after infection. In this time some 15 daughter RF molecules accumulate. Although they first appear in the open form they are converted within a minute to the closed or supercoiled form, so that by 12 minutes the large majority of RF molecules are in the closed form.

The daughter RF molecules remain free of the membrane fraction unless the cell has more than one site; in this case the secondary site may be colonized by a daughter RF which can in turn start to replicate.

The second phase of RF replication ends at about 12 min after infection. At this time host DNA synthesis which had continued at its preinfection rate ceases. Net RF synthesis also ceases although a low level of RF replication at the site may persist for a time. Synthesis of progeny single-strand DNA begins at a rate equivalent to 5-10 times the previous rate of RF synthesis.

To initiate this third phase of DNA replication (progeny DNA synthesis) the RF molecules which have accumulated are converted within a few minutes to an open form, in all of which the viral strand is open and the complementary strand closed. All of these RF molecules are then used in an asymmetric semiconservative synthesis in which the viral strand is displaced from the RF into a virus particle while a new viral strand is laid down on the complementary ring. This process proceeds at a rate of approximately one new viral strand per RF per minute. Obviously the displaced viral strand must be closed at some time, as it is known to be closed in the mature virus particle.

Free single-strand rings of DNA are never found. Unless functional coat proteins of cistrons III and IV are both present the single-stranded progeny DNA is not made. The coat protein evidently plays a regulatory role as well as a structural role, and mutants are known in the coat protein cistron which

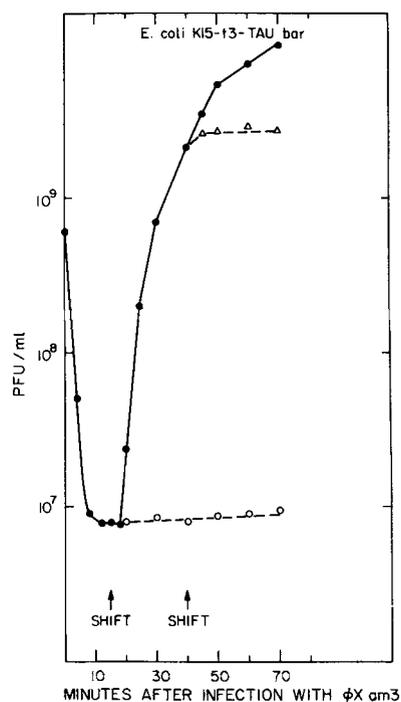


FIGURE 2. A culture of *E. coli* strain 15t3-TAU bar was grown at 30°C to 4.5×10^8 /ml, infected with ϕX am3 (phage multiplicity, $m = 2$) and divided into three portions. Infective centers were measured (by artificial lysis) at various times after infection in each portion.

- — ● This portion was maintained at 30°C.
- — ○ The temperature of this portion was raised to 42°C at 12 min after infection (prior to the close of the eclipse period).
- △ — △ The temperature of this portion was raised to 42°C at 40 min after infection (during formation of progeny phage).

produce progeny DNA and particles at 2-3 times the rate of wild type.

It has been a long-standing question whether the viral DNA actually penetrates the cell as a single-stranded ring or whether it might be in effect pulled into the cell as it became an RF. With the aid of a host mutant known to be temperature-sensitive for DNA synthesis it has been possible to demonstrate that the free viral single-stranded rings are indeed released from the virus in the absence of DNA synthesis.

If a temperature-sensitive mutant of the strain 15 TAU-bar, originally isolated by Rasmussen and Weywadt and given to us by Dr. Phillip Hanawalt, is infected with ϕX at the high temperature, no phage are produced (Fig. 2). If, during the period of phage production in this host, the temperature is raised to the restrictive level, ϕX synthesis abruptly stops. Upon infection of such a strain with ϕX at the high temperature no RF is formed (Fig. 3). (An analogous result was shown earlier in a Bonhoeffer mutant by Dr. David Denhardt.)

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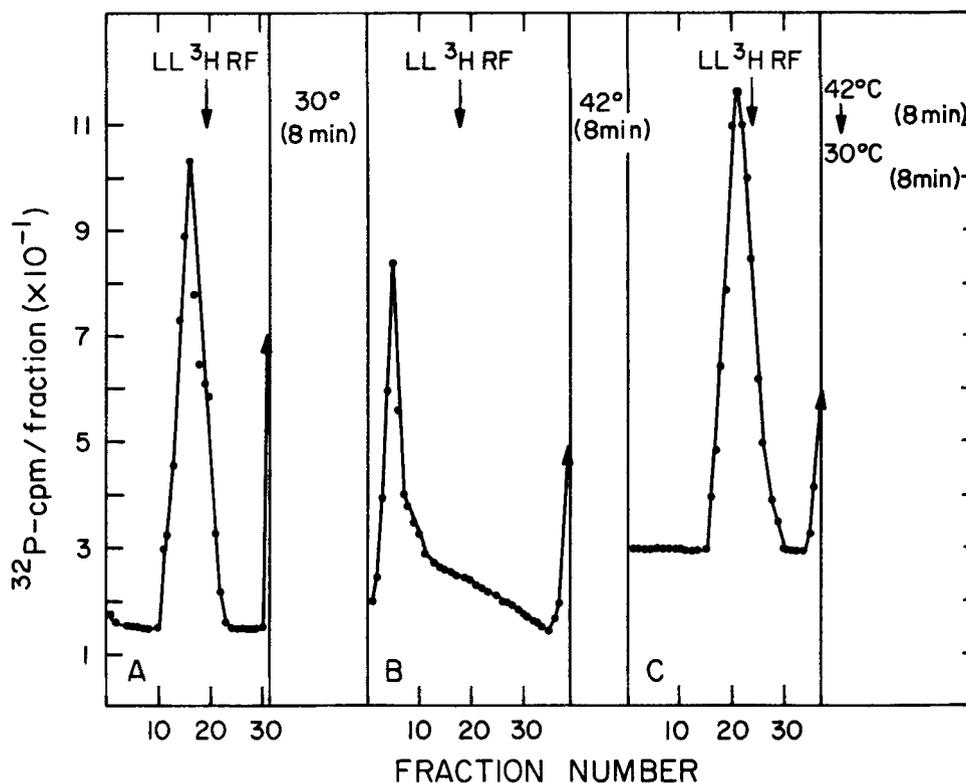


FIGURE 3. A culture of *E. coli* strain 15t3-TAU bar was grown at 30°C to 4×10^8 /ml and divided into three portions. Each portion was separately infected with $^{13}\text{C}^{15}\text{N}^{32}\text{P}$ - ϕ X ($m = 5$).

(A) This portion was maintained at 30°C. At 8 min after infection, cells were lysed with lysozyme-EDTA. The lysate was sheared by passage through a syringe and then centrifuged to equilibrium in a neutral CsCl density gradient.

(B) The temperature of this portion was raised to 42°C before infection. At 8 min after infection, a lysate was prepared as in part A and centrifuged to equilibrium in a neutral CsCl density gradient.

(C) The temperature of this portion was raised to 42°C before infection. At 8 min after infection the temperature was reduced to 30°C. At 16 min after infection, a lysate was prepared as in part A and centrifuged to equilibrium in a neutral CsCl density gradient.

In each gradient the arrow marks the location of ^3H -labeled RF (without density label).

However, under these conditions free single-stranded DNA is present in the cell. If the temperature is dropped these are then converted to RF. It is interesting to speculate upon what cellular component is involved here, since such cells are known to perform DNA repair at the restrictive temperature.

The result of another experiment to demonstrate the failure of daughter RF molecules to replicate during the period of RF replication is presented in Fig. 4 (see also Stone, 1967). Cells were infected with ^{32}P labeled phage in heavy ($^{13}\text{C}^{15}\text{N}$) medium in the presence of ^3H -thymine. After four minutes a sample was taken for DNA extraction and the remainder transferred to light ($^{12}\text{C}^{14}\text{N}$) medium in an excess of cold thymine. After seven more minutes an extract of DNA was made from these cells.

After four minutes fully heavy progeny DNA containing ^3H and hybrid density parental DNA containing both ^3H and ^{32}P are present. After transfer to light medium and seven minutes' further incubation, about half of the parental DNA

has shifted (the input multiplicity was 3) to the fully light position. Infectivity data indicate a synthesis of fully light progeny DNA. But the thymine-labeled progeny DNAs, made in heavy medium, remain at the full heavy density indicating their failure to replicate.

Experiments carried out in our laboratories and others some years ago, intentionally done at a low multiplicity of infection, indicated a failure of transfer of parental DNA to progeny (Sinsheimer, 1961; Kozinski, 1961). This result is now explained in our model as a consequence of the persistence of the input viral strand at the membrane site. If, however, a considerable multiplicity of input phage were used, one might expect that at least some of the RF molecules formed would join the progeny RF pool and be utilized, thereby contributing their viral strands to progeny. Indeed this can happen. In multiplicities of 10 or above, transfer of parental strands through RF to progeny can be demonstrated. That such high multiplicity is needed is a consequence of a poorly understood but

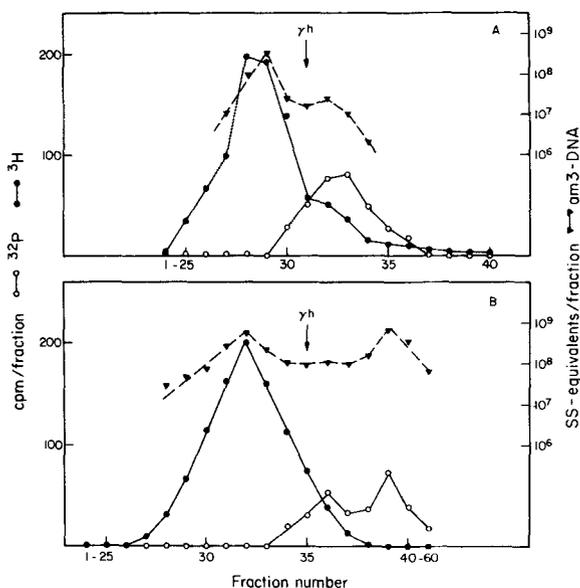


FIGURE 4. Cells of *E. coli* strain HF4704 were grown to 3×10^8 /ml in $^{13}\text{C}^{15}\text{N}$ medium and infected in this medium with ^{32}P - ϕX ($m = 3$) in the presence of ^3H -thymine. At four min after infection, DNA was extracted from one portion of the culture. Cells from the other portion were transferred to $^{13}\text{C}^{14}\text{N}$ medium with an excess of unlabeled thymine and incubated for another seven min. After this, DNA was extracted from this portion.

(A) Distribution in a neutral CsCl density gradient of ^{32}P , ^3H and infectivity of the DNA extracted after 4 min.

(B) Distribution in a neutral CsCl density gradient of ^{32}P , ^3H and infectivity of the DNA extracted after the additional 7 min incubation.

In both gradients the arrow indicates the location of marker single-stranded viral DNA.

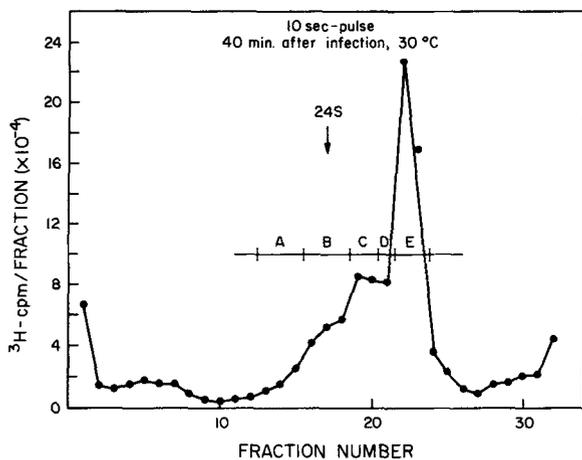


FIGURE 5. Cells of *E. coli* strain CH502 (*uvr*⁻, *DNase I*⁻, *thy*⁻) were grown at 30°C to 1.2×10^8 /ml and infected with ϕX *am3* ($m = 6$). After 40 min of infection, ^3H -thymidine was added. Ten seconds later the culture was abruptly chilled. DNA was extracted, sedimented through a neutral sucrose gradient (0.3 M NaCl, Tris-EDTA) and the distribution of ^3H -label determined.

The arrow indicates the portion of a marker of infective single-stranded ϕX DNA.

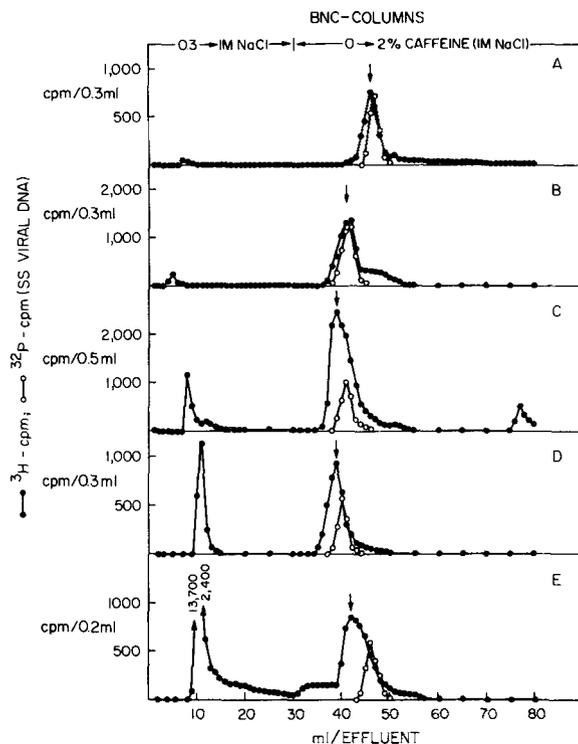


FIGURE 6. The fractions of Fig. 5 were pooled into five groups, A-E. The DNA in each group was then fractionated on a column of benzoylated, naphthoylated DEAE-cellulose (2 cm \times 1.5 cm diam.) and the distribution of ^3H -label determined. A marker of ^{32}P -labeled ϕX viral DNA was added to each DNA sample before fractionation.

The fractions indicated by arrows in each gradient were further analyzed as shown in Figure 7.

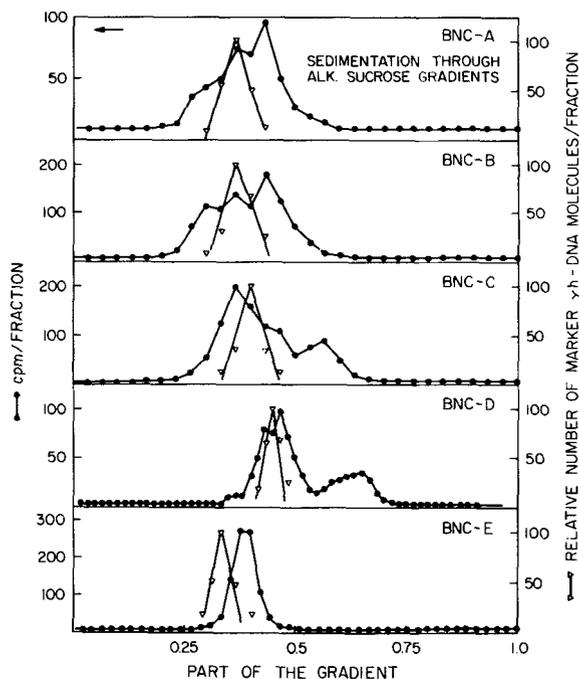


FIGURE 7. Peak fractions from the column eluate of Fig. 6 (indicated by arrow) were sedimented through a sucrose gradient at pH 12.5 (measured) and the distribution of ^3H (pulse-label) and of ^{32}P (added ϕX DNA marker) determined.

well-observed phenomenon whereby parental RF molecules formed from input DNA are rather firmly attached to the membrane fraction even if they are not at a site at which they can replicate. This is in contrast to progeny RF, which rarely attach to membrane. Such parental RF only occasionally and slowly become detached to join the progeny RF pool which can contribute DNA to progeny phage. Thus at an input multiplicity of 33 (light ^{32}P -phage infecting cells in heavy medium), after 10 minutes 90% of the ^{32}P of the parental phage was in RF. After 3 hr this was reduced to 60% and ^{32}P corresponding to 11 viral DNAs was found in virus particles of hybrid density (light DNA, heavy coat).

More recently we have attempted to observe possible intermediates in the synthesis of the progeny single-strands by reducing the effective length of pulse both by decreasing the absolute time and by lowering the temperature. Thus Fig. 5 indicates the sedimentation pattern of the radioactivity incorporated during a 10-sec pulse of ^3H -thymine at 30°C at 40 min after infection. The arrow indicates the position of single-stranded DNA.

The fractions from the gradient were divided into five groups, A-E as shown. These were then analyzed on a column of benzoylated-naphthoylated DEAE cellulose which has previously been shown to fractionate nucleic acids according to their content of exposed purine and pyrimidine rings—or roughly according to their content of single-stranded DNA (Tener et al., 1966; Sedat et al., 1967).

As can be seen in Fig. 6, the fastest sedimenting fractions cling most tenaciously to the column (almost as tightly as does a viral ϕ X DNA marker, suggesting the presence of a free single-strand portion comparable in length to a viral DNA) while the slower sedimenting portions split into two fractions—an increasing proportion of pure RF and a decreasing proportion of a fraction that behaves as though it contained shorter single-stranded regions.

When the peak fractions from each of the columns were analyzed by sedimentation in alkali (Fig. 7), it became clear that almost all of the original A-E fractions contained labeled components which after denaturation sedimented faster than either viral rings or linear single-stranded DNA of viral DNA length. This might be expected from a mode of replication which added on to the 3' end and displaced the prior viral strand as it grew.

However, several fractions also clearly contain well-defined components smaller than the open viral single-strands. While one can formulate ad hoc hypotheses to account for these results they contain

just about as many assumptions as facts. It is clear that more detailed experiments are needed.

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