Analysis of the \( \phi X \) DNA replication cycle by electron microscopy

(rolling circle intermediate/partially duplex ring/synthesis of positive strand DNA/synthesis of negative strand DNA/synthesis of single-stranded and double-stranded DNA circles)

KIRSTON KOTHS AND DAVID DRESSLER

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT We have monitored the development of intracellular \( \phi X \) DNA forms during the course of a virus life cycle that duplicates as closely as possible the normal infection of individual cells by single virions. The viral DNA was isolated in a one-step purification procedure, and quantitative electron microscopy was performed on the samples, resulting in the following conclusions:

(i) Early in the life cycle, when the cells accumulate duplex rings, two types of DNA replication intermediates are observed: a rolling circle with a single-stranded tail; and a novel form, a single-stranded circle that is partially duplex. Thus, duplex ring synthesis appears to occur in two asymmetric steps, with possible strand DNA first being processed from the tail of the rolling circle and circularized, before it acts as a template for negative strand synthesis.

(ii) Late in the life cycle, as single-stranded circles are synthesized and virus particles are assembled, only one replicating intermediate is observed—the rolling circle with a single-stranded tail. At this stage, the number of rolling circles reaches a level of about 35 per cell.

(iii) The net rate of polymerization in the rolling circle intermediates is about 200 nucleotides per sec.

\( \phi X \) is a minute virus with only minimal autonomy. It relies almost entirely on host cell gene products for the replication of its DNA. These host cell enzymes are the same ones that are involved in the replication of the bacterial chromosome. For this reason, studies of the structure of intracellular viral DNA forms are valuable because they provide descriptions of natural substrates upon which the DNA replication enzymes work.

In this paper we present an electron microscopic analysis of the development of intracellular \( \phi X \) DNA forms during the course of an infection that reflects as closely as possible the normal infection of individual cells by single virions.

Experimental design

The basic requirements for an isolation procedure that will allow quantitative electron microscopic study of intracellular viral DNA forms are: (i) effective separation of viral from host DNA, (ii) efficient recovery of all forms of viral DNA, and (iii) preservation of the structural integrity of the viral DNA molecules. To satisfy these requirements as closely as possible, we have modified a modification of the density shift protocol of Melsens and Stahl (1–3).

The present method involves a minimum of handling of the sample and yields intracellular viral DNA sufficiently pure for direct examination in the electron microscope after a single purification step. Host cells are grown in heavy isotope medium, then transferred to light medium, and infected with isotopically light phase. During the subsequent incubation period the bacterial DNA remains heavy or shifts toward hybrid. In contrast, as they develop, the intracellular \( \phi X \) DNA forms have densities that are essentially light. Separation of the viral DNA forms from the host DNA may then be accomplished by centrifugation of the total cell lysate in a neutral CsCl equilibrium gradient.

Infection. Fig. 1 shows the development of intracellular virus particles during the \( \phi X \) infection. The extent and kinetics of phage production indicate that the presence of heavy isotopes during the preliminary growth of the host cells has no adverse effect on the subsequent viral life cycle; the final burst size is >1000 phage per infected cell.

At the times indicated by the arrows in Fig. 1, aliquots of the infected culture werepulse-labeled with \( ^{3}H \)thymidine, the cells were harvested and lysed, and the total cell lysates were centrifuged to equilibrium in CsCl.

Typical gradients obtained from the lysates are shown in Fig. 2. Two broad peaks of radioactivity are seen. The denser peak, which is an area of high viscosity, contains the vast bulk of the host DNA; the lighter peak contains the viral DNA forms, which we have examined in detail.

Quantitation of \( \phi X \) DNA forms. The above protocol concentrates and purifies the viral DNA, making it possible to use the electron microscope to determine both the relative and absolute numbers of \( \phi X \) forms as the virus life cycle develops. This may be accomplished by adding a known amount of an internal standard molecule to the sample and then spreading the material for electron microscopy. The internal standard chosen was a circular supercoiled plasmid DNA that could be readily distinguished from \( \phi X \) DNA forms in the electron microscope because of its size (11.4 x 10^9 daltons).

In a typical analysis, 1/2500 of a CsCl-purified \( \phi X \) DNA sample (2 \( \mu l \)) was supplemented with 8 x 10^8 plasmid molecules and spread for the electron microscope, as described in the legend to Fig. 3. A random sampling of 1000 plasmid DNA molecules was then counted along with the number of each \( \phi X \) DNA form. Then, for instance, when the number of \( \phi X \) rolling circles was 180 in the material harvested at 50 min, it could be concluded that the sample aliquot contained 18% as many rolling circles as plasmid molecules, or 1.4 x 10^8 rolling circles. Because the sample spread for electron microscopy represented 1/2500 of the material from the CsCl gradient and had been derived from 10^10 infected cells, it could be calculated that the number of rolling circle intermediates was (1.4 x 10^8) (2500) rolling circles + 10^10 cells, or 35 rolling circles per cell.

About 100 such spreads and molecule counts were made in this set of experiments (Table 1), and calculations similar to the one described above were made to determine the number of intracellular \( \phi X \) DNA forms as a function of time after infection.

Duplex rings

The traditional view of the \( \phi X \) DNA replication cycle as a period of double-stranded circle synthesis followed by a period...
of single-stranded circle synthesis (8) is quantitatively confirmed by the data of Table 1 and Fig. 4. Duplex rings were the net product of DNA synthesis early in the phage life cycle. The number of double-stranded DNA circles increased during the first 25 min of infection, reaching a plateau level of about 60 per cell.

The duplex rings are of two kinds, occurring in approximately equal numbers. One type is a double-stranded DNA circle (Fig. 3C) in which both strands are covalently closed (9) and superhelical twists have been introduced into the molecule by DNA gyrase (10). The other form is a relaxed duplex ring whose open appearance is the result of either an internucleotide bond interruption or a deficiency of superhelical twists (Fig. 3B). About 2% of the duplex rings are dimeric in size (Fig. 3F) and are probably the product of genetic recombination (11, 12). From the point of view of replication, all of these ΦX species are resting.

Rolling circles

Duplex rings that are actively engaged in DNA synthesis are detected almost as soon as ΦX DNA forms can be seen. This form, the rolling circle intermediate, was shown in earlier experiments to consist of a circular negative strand that serves as a template for the synthesis of a longer-than-genome-length positive strand (4, 13–17). The growing positive strand is positioned so that its 3'-OH end lies on the template ring and is thus in a position to be continuously elongated by the DNA replication machinery. Material for progeny circles is derived from the tail of the rolling circle intermediate.

The rolling circle intermediates increased from a level of about 1 per cell at 5 min to about 35 per cell at 40 min. Well over 1000 rolling circle intermediates were observed in this study, and more than 100 were randomly chosen, photographed, and analyzed, giving rise to the following observations.

\[ \text{Fig. 1. Infection and growth of phage. Prior to infection, } \text{Escherichia coli strain HP 4704 (thy},^-\text{, su}^-\text{, uvr }A^-\text{) was grown at 30°} \text{ in heavy isotope medium containing } ^{15}\text{NH}_4\text{Cl and } ^3\text{H}_2\text{O(3). When the cells reached a titer of } 4 \times 10^9 \text{ per ml, they were harvested by centrifugation, resuspended in light isotope medium, incubated for 15 min, and then infected with } \text{ΦX amber 3, a mutant phage isolated by Hutchison and Sinsheimer that is defective only in cell lysis. A multiplicity of infection of 0.7 phage per cell was chosen to ensure that the majority of cells would be infected only once. The rate of phage production was linear after about 25 min, with about 10 plaque-forming phage being matured per minute per infected cell. [Additional points (C) are from a repeat of this experiment.] No progeny phage were produced in a portion of the culture that received chloramphenicol (35 μg/ml) 10 min prior to infection (Δ - Δ).} \]

\[ \text{Rolling circles were seen throughout the DNA replication cycle, both early during the period of duplex ring synthesis and later when single-stranded circles are the product of replication.} \]

\[ \text{(ii) During both duplex ring synthesis and single-stranded circle synthesis, the rolling circles had tails that were single stranded. Only occasionally (5/1000) was a rolling circle observed in which the tail had been converted to the duplex state (Fig. 5).} \]

\[ \text{(iii) The lengths of the tails of the rolling circles varied randomly from a few hundred nucleotides (the shortest length measurable in the electron microscope) to about unit length, 5400 nucleotides (Fig. 6). This result indicates that the maturation of DNA from the tail of the intermediate occurs rapidly; for instance, as soon as the proper nicking sequence has been exposed in the tail or by repeated nicking of the positive strand on the duplex ring at the origin of replication. Either event would permit the separation of a unit viral genome from the intermediate each time the growing point approaches the origin.} \]

\[ \text{(iv) Usually the rolling circles consisted of a single-stranded tail emerging from a monomer-size duplex ring. However, in about 5% of the rolling circles, the tail was being synthesized by copying around a dimeric template ring (as in Fig. 3G). Even in these cases, however, the emerging tails had the same length distribution as those being synthesized from monomer rings (Fig. 6). This result offers further evidence in support of the rapid maturation of progeny DNA from the tail of the rolling circle intermediate. Additionally, this result provides a mechanism whereby, through replication, recombinant progeny genomes can be matured from a genetically heterozygous dimer (11, 12) that has been formed previously by recombination between two monomer rings.} \]

\[ \text{(v) The single-stranded tails of the rolling circle intermediates were composed of positive strand material. This result is in accord with previous pulse-labeling studies (4, 13–17). It has been determined independently in the present experiments by virtue of the ability of the tails to hybridize a defined piece of ΦX negative strand DNA (derived from a restriction enzyme digestion of purified duplex rings). The hybridization of the negative strand fragment creates a duplex region on the tail which can be observed in the electron microscope (Fig. 7).} \]
FIG. 3. Electron microscopy. Intracellular $\phi X$ DNA forms, representing various stages in the virus life cycle, were recovered from CsCl gradients (see the pooled fractions in Fig. 2) and spread for electron microscopy without further processing. This protocol was designed to omit further processing steps such as dialysis and ethanol precipitation which involve loss of DNA. Control smears of samples containing known amounts of single-stranded and double-stranded $\phi X$ circles showed that both forms were equally well retained on the grids. Thus we think we have minimized, although one can never eliminate, the possibility of selective loss of various DNA forms. Typically, the hyperphase consisted of 2–8 $\mu l$ of CsCl/DNA solution mixed with 2 $\mu l$ of internal standard DNA ($8 \times 10^6$ molecules of plasmid pKB-105; concentration determined by OD$_{260}$ after CsCl velocity gradient sedimentation and, independently, with the model E ultracentrifuge). The DNA sample was then supplemented with 2.5 $\mu l$ of cytochrome c solution (1 mg/ml in 1 M Tris/100 mM EDTA, pH 8.6), 18 $\mu l$ of double-distilled, deionized water, and 20 $\mu l$ of formamide. One-third of the 50–$\mu l$ hyperphase was spread onto a 27 ml hypophase of 8% formamide/1 mM Tris/0.1 mM EDTA, pH 8.5. The DNA–protein film was adsorbed to a Parlodion-coated 200-mesh copper grid. The sample was then dehydrated by immersion in 90% ethanol for 3 sec and stained for 30 sec in uranyl acetate (50 mM in 90% ethanol/0.5 M HCl). The grids were examined in a Philips 300 electron microscope with dark-field illumination. Because samples could be spread directly from the CsCl gradients and shadowing was not necessary to enhance contrast (3), the total time required for grid preparation was about 10 min. The use of formamide in the spreading solutions allows single-stranded DNA to exist in an extended rather than a collapsed state. Furthermore, the single-stranded DNA appears thinner and less rigid than duplex DNA (6, 7). (A) Single-stranded circle. (B) Relaxed duplex ring. (C) Supercoiled duplex ring. (D) Rolling circle intermediate. (E) Partially duplex ring (arrows show extent of duplex region). (F) Dimer-size duplex ring. (G) Rolling circle intermediate in which the template ring is a dimer circle.

We interpret the above data to indicate that, throughout the $\phi X$ life cycle, rolling circle intermediates produce positive strand material that is rapidly detached from the tails to yield single-stranded rings.

This positive strand material is packaged directly into coat protein late in the life cycle. Earlier it must be used for the synthesis of $\phi X$ duplex rings. To this end it seemed reasonable to suppose that duplex ring synthesis involves two asymmetric events—the initial production of single-stranded circles from the rolling circle intermediates, followed by the conversion of these circles to a duplex state via the synthesis of a complementary strand.

**Partially duplex rings**

The above model predicts that, during the period when the number of duplex rings is increasing, one may hope to see sin-

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**Table 1. $\phi X$ DNA forms as they appear with time after infection**

<table>
<thead>
<tr>
<th>Time after infection, min</th>
<th>Single-stranded circles</th>
<th>Duplex rings</th>
<th>Relaxed</th>
<th>Rolling circles</th>
<th>Partially duplex rings</th>
<th>Dimeric forms</th>
<th>Other</th>
<th>Total $\phi X$</th>
<th>Total internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15</td>
<td>266</td>
<td>407</td>
<td>73</td>
<td>10</td>
<td>17</td>
<td>2</td>
<td>790</td>
<td>1200</td>
</tr>
<tr>
<td>25</td>
<td>868</td>
<td>212</td>
<td>350</td>
<td>172</td>
<td>1</td>
<td>26</td>
<td>5</td>
<td>1634</td>
<td>1500</td>
</tr>
<tr>
<td>35</td>
<td>1898</td>
<td>137</td>
<td>194</td>
<td>185</td>
<td>1</td>
<td>17</td>
<td>2</td>
<td>2434</td>
<td>1000</td>
</tr>
<tr>
<td>50</td>
<td>3454</td>
<td>136</td>
<td>248</td>
<td>180</td>
<td>0</td>
<td>13</td>
<td>1</td>
<td>4032</td>
<td>1000</td>
</tr>
<tr>
<td>65</td>
<td>4746</td>
<td>92</td>
<td>201</td>
<td>159</td>
<td>1</td>
<td>18</td>
<td>0</td>
<td>5217</td>
<td>1000</td>
</tr>
</tbody>
</table>

Cells grown in 35 $\mu g/ml$ chloramphenicol

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>Single-stranded circles</th>
<th>Duplex rings</th>
<th>Relaxed</th>
<th>Rolling circles</th>
<th>Partially duplex rings</th>
<th>Dimeric forms</th>
<th>Other</th>
<th>Total $\phi X$</th>
<th>Total internal standard</th>
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<tr>
<td>38</td>
<td>3697</td>
<td>995</td>
<td>100</td>
<td>55</td>
<td>111</td>
<td>4</td>
<td>5000</td>
<td>7792</td>
<td></td>
</tr>
</tbody>
</table>

Duplex rings with two single-stranded tails attached to the circle at the same point were considered to be rolling circles in the process of branch migration. The dimer category includes open and closed dimeric circles, and also dimer rolling circles, interlocked monomers, and figure-8 molecules. The category "other" includes such structures as rolling circles with double-stranded tails, but not Cairns forms (none of which were seen in this study). In all cases, the number of unscorable, tangled molecules was less than 2%. The data for $\phi X$ forms produced in chloramphenicol-treated cells represent the total for three time points: 26, 41, and 55 min. This experiment has been done three times at 30° and once at 37°, with equivalent results.
single-stranded circles in which a complementary strand has begun
to be laid down. We have in fact observed such partially duplex rings
(Fig. 3E). In the 100 such molecules we have observed, the percentage of the circle that is duplex ranged from 10 to
90%.

Early during the φX life cycle, rolling circle intermediates
with single-stranded tails and partially duplex circles are both observed (Table 1). Operating together, these two structures appear to form the complementary pair of replicating intermediates necessary for duplex ring synthesis.

Because the number of partially duplex rings observed at
early times in the φX infection is low (one to three per cell), one
must be concerned that they might just represent the conversion of
late-infecting φX single-stranded rings to the duplex state—that is, the formation of the first φX double-stranded
circle. If this were so, these structures might only represent the
initial stage of φX DNA replication and have nothing to do with
duplex ring synthesis in general. To obtain further evidence as
whether the partially duplex circles are involved in duplex ring synthesis, we took advantage of the fact that in the presence of chloramphenicol the accumulation of φX single-stranded rings late in the life cycle does not occur and duplex ring synthesis continues for several hours (8, 18) (see also Table 1). It is
thus possible to harvest cells synthesizing duplex rings long after
it would be reasonable to expect single-stranded circles to enter

![Fig. 4. φX DNA forms per cell, calculated from the data of Table 1. The 8-min points are from an identical experiment, which gave the same results.](image)

![Fig. 5. A rare φX rolling circle intermediate with a double-stranded tail.](image)

![Fig. 6. Lengths of the single-stranded tails of rolling circles measured from photographs of monomeric (---) and dimeric (-----) rolling circles. The length ratio of single-stranded to double-stranded DNA in our spreadings was 0.94. The tail lengths shown have been corrected for this slight shrinkage factor.](image)

![Fig. 7. A defined fragment of the φX negative strand (Z3, purified from duplex rings by digestion with the restriction enzyme Hae III) was hybridized to the rolling circles. The rolling circle tails were able to anneal the isolated negative strand fragment (but not its positive strand counterpart). This shows that the tail is composed of positive strand material. Furthermore, the unique location of the duplex region on the tail can be used to define the location of the end of the tail—the origin-terminus of the φX positive strand synthesis (in the Hae III fragment Z6b).](image)
not to pause for long periods at particular locations on the template duplex ring.

If each rolling circle is associated with a DNA polymerase system, then the number of DNA polymerase III molecules per cell must be at least 35.

Discussion

This paper has presented an overview of the φX DNA replication cycle as seen by electron microscopy. In particular we have followed and quantitated the increase in concentration, per cell, of the naturally occurring viral DNA forms. The results indicate that rolling circle intermediates with a single-stranded tail operate throughout the viral life cycle. The observation of a novel φX form, the partially duplex ring, allows us to explain how a rolling circle generating single-stranded circles participates in duplex ring synthesis. The overall replication scheme is summarized in Fig. 8. It is based on DNA forms observed through electron microscopy and in vitro pulse-labeling studies.

Our results are in full accord with the powerful in vitro studies of Kornberg and Hurwitz and their colleagues (20–22). Using purified cell proteins and one virus-coded enzyme, they have been able to transform duplex rings into rolling circles in vitro.

Both the in vivo and in vitro data at the present time provide strong support for the proposals of the rolling circle model (13, 4, 15, see also ref. 23) that (i) both the early synthesis of duplex rings and the later synthesis of single-stranded circles can be understood in terms of one replication strategy, and (ii) φX positive strands and negative strands are synthesized in two asymmetric events—the positive strand being elongated continuously after creation of a 3'-OH primer group by nicking of a covalently closed φX duplex ring, and the negative strand being elongated discontinuously (that is, by de novo initiation of a DNA fragment on a segment of a single-stranded template DNA).

The relatively large number of rolling circle intermediates recovered per cell in our experiments (about 35) is encouraging from the point of view of studying these actively replicating DNA molecules biochemically. Indeed, there is at this time no other experimental system in which a uniform replicating intermediate can be isolated in such quantity.

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