Bacteriophage ϕ X174 RF DNA Replication *in Vivo* A Study by Electron Microscopy

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We have investigated bacteriophage ϕ X174 RF $\dagger$ DNA replication by electron microscopy. Three different types of replicative intermediates were observed: rolling circles, partially duplex DNA circles and structures consisting of two DNA circles connected at a single point.

Rolling circles with a single-stranded or partially double-stranded DNA tail were both observed. After cleavage of the rolling circles with the restriction endonuclease from *Providentia stuartii* 164 (*Pst*I) the startpoint of rolling circle replication could be located at 21 map units from the *Pst*I cleavage site in agreement with the previously determined position of the origin of ϕ X RF DNA replication.

Partially duplex DNA circles consist of circular viral DNA strands and incomplete complementary DNA strands. After cleavage of these molecules with *Pst*I information about the startpoints of the synthesis of the complementary DNA strand was obtained.

The connected DNA circles always contain one completely double-stranded DNA circle whereas the other circle consists of either single-stranded, partially duplex or completely duplex DNA.

Part of the duplex-to-duplex DNA circles represent the well-known figure eight or catenated circular dimers. The other connected DNA circles presumably represent replication intermediates which arise by the association of the end of the genome length tail of the rolling circle with the origin-terminus region. This is suggested by the fact that the point of contact between the two DNA circles is located at approximately 21 map units from the *Pst*I cleavage site, i.e. at the origin-terminus region of the ϕ X genome. The connected DNA circles may be intermediates in the circularization and cleavage of the genome-length tail of the rolling circles *in vivo*.

A model for ϕ X174 RF DNA replication *in vivo* summarizing the data obtained by biochemical (Baas *et al.*, 1978) and electron microscopic analysis of replicative intermediates is presented (Fig. 9).

1. Introduction

ϕ X174 is a small DNA-containing bacteriophage. Its circular single-stranded DNA replicates *via* a circular double-stranded replicative form as was first shown by Sinsheimer *et al.* (1962).

ϕ X174 RF $\dagger$ DNA replication starts with the introduction of a single nick in the viral DNA strand by the phage coded gene *A* protein (Francke & Ray, 1971, 1972).

$\dagger$ Abbreviations used: RFI, replicative form DNA with both strands closed; RFII, replicative form DNA with one or more discontinuities in either strand; PrI₂, propidium diiodide.

The position of this nick has been located at a specific site within gene *A* by studies *in vivo* (Baas & Jansz, 1972; Baas *et al.*, 1976) and *in vitro* (Ikeda *et al.*, 1976; Eisenberg *et al.*, 1977). Recently, Langeveld *et al.* (1978) have shown by experiments *in vitro* that the viral DNA strand of ϕ X174 RFI DNA is cleaved by the gene *A* protein exposing a 3'-OH bounded G residue at position 4305 of the ϕ X174 DNA sequence (Sanger *et al.*, 1978). Position 4305 is identical to position 4297 of the provisional ϕ X DNA sequence (Sanger *et al.*, 1977). According to the rolling circle model for ϕ X174 RF DNA replication the 3' end of the viral DNA strand is then elongated continuously thereby displacing the 5' end as a linear single-strand DNA tail (Gilbert & Dressler, 1968). These rolling circles have been identified by biochemical studies (Francke & Ray, 1971; Schröder & Kaerner, 1972; Schröder *et al.*, 1973; McFadden & Denhardt, 1975; Fukuda & Sinsheimer, 1976; Baas *et al.*, 1978) and by electron microscopy (Knippers *et al.*, 1969; Dressler, 1970; Francke & Ray, 1972; Koths & Dressler, 1978). The studies of Eisenberg *et al.* (1977) *in vitro* present evidence for a looped rolling circle, i.e. the 5' end of the DNA tail travels along with the replication fork.

Another type of replicative intermediate consisting of circular viral DNA strands and incomplete complementary DNA strands has been identified by Schekman *et al.* (1971) and by Eisenberg & Denhardt (1974*a,b*). These molecules probably arise as products of rolling circle DNA replication. The circularization of the displaced genome length single-stranded or partially double-stranded DNA tail yields circular viral DNA strands or RFII molecules with a closed viral DNA strand and multiple gaps in the complementary DNA strand (Eisenberg & Denhardt, 1974*a,b*). The other daughter molecule has a circular complementary DNA strand and a small gap in the viral DNA strand which is located at the origin-terminus region of the ϕ X genome (Eisenberg *et al.*, 1975; Baas *et al.*, 1978).

We have analyzed rolling circles and partially duplex ϕ X174 DNA circles in the electron microscope before and after digestion with the restriction endonuclease from *Providentia stuartii* (*Pst*I). This enzyme cleaves ϕ X174 RF DNA only once at position zero of the Sanger map of ϕ X174 DNA (Sanger *et al.*, 1978). The results identify a unique startpoint of rolling circle DNA replication and at least six startpoints for the synthesis of the complementary DNA strand on the physical map of ϕ X174 DNA.

In addition to rolling circles and partially duplex DNA circles we have observed a new type of ϕ X DNA replicative intermediate consisting of one double-stranded and one (partially) single-stranded DNA circle connected to each other at one point. Analysis of these structures after *Pst*I cleavage revealed that the two DNA circles are connected to each other at the origin-terminus region of the ϕ X genome. The results suggest that these structures are intermediates in the circularization and cleavage of the genome-length tail of the rolling circle.

2. Materials and Methods

(a) Infection, labeling and lysis of cells

Escherichia coli H502 was grown at 37°C in 11 TPG medium (Levine & Sinsheimer, 1968), containing 200 μ g Casamino acids/ml and 2 μ g thymine/ml. At 4×10^8 cells/ml the cells were collected by centrifugation and resuspended in 80 ml fresh medium. In order to suppress bacterial DNA synthesis, the cells were treated with 50 μ g mitomycin C/ml (Sigma Chemical Comp.) for 10 min at 37°C (Lindqvist & Sinsheimer, 1967). To remove mitomycin C the cells were pelleted again and suspended in 900 ml prewarmed (30°C) TPG medium. The concentration of thymine in the TPG medium was lowered to 0.5 μ g/ml in order to enhance the specific activity of the label. After 2 min at 30°C, 20 ml

of chloroamphenicol (2 mg/ml) was added to the cells and 5 min later the culture was infected with ϕ X, suspended in 50 ml TPG medium, with a multiplicity of infection of 5. Then 20 min after infection, 500-ml portions of the culture were pulse-labeled with 5 mCi [methyl- 3 H]thymidine (40 to 60 Ci/mmol; Radiochemical Centre, Amersham, U.K.), dissolved in 25 ml of TPG. The pulse was stopped by pouring the infected culture into a stainless steel beaker containing crushed ice, made from 600 ml Tris/EDTA buffer (0.05 M-Tris, 0.005 M-EDTA, pH 8.0) and a solution of 380 ml Tris/EDTA buffer, 15 ml of 5 M- NaN_3 and 5 ml of 1.5 M-KCN at 0°C. The beaker was immediately swirled around in a solid CO_2 /acetone bath. After thawing, the cells were harvested and washed 3 times with 200 ml borate/EDTA/ NaN_3 /KCN in the cold. A 250 ml portion of the culture was resuspended at 0°C in a Sorvall SS34 centrifuge tube in 20 ml of 10% (w/v) sucrose, 0.05 M-Tris (pH 8.0). Then 2.5 ml of lysozyme (10 mg/ml in 0.25 M-Tris, pH 8.0) and 12 ml of 0.25 M-EDTA (pH 8.0) were added to the cells. After 30 min at 0°C, 0.8 ml of Pronase (Calbiochem, B grade; 10 mg/ml in 0.05 M-Tris, pH 8.0, autodigested for 2 h at 37°C) and 2.5 ml of a 20% (w/v) solution of sodium dodecyl sulphate were added to the lysate by blowing out a pipette at the bottom of the centrifuge tube. The centrifuge tube was placed in a waterbath at 37°C and after 10 min the clear viscous lysate was placed in the cold room without cooling (4°C). After 12 to 16 h, *E. coli* DNA together with sodium dodecyl sulphate were precipitated for 30 min at 14,000 revs/min in a Sorvall SS34 rotor. The supernatant solution was dialyzed against Tris/EDTA buffer and extracted twice with freshly distilled phenol. The phenol contained 0.1% hydroxyquinoline and was equilibrated with Tris/EDTA buffer. Nucleic acids were precipitated by the addition of 0.1 vol. 3 M-sodium acetate (pH 5.5) and 2 vol. isopropanol, followed by refrigeration overnight at -20°C. Nucleic acids were dissolved in Tris/EDTA buffer, after dialysis against the same buffer, treated with 50 μ g RNase/ml (Worthington Biochemical Corporation; preincubated for 10 min at 80°C) for 10 min at 37°C and layered directly on top of 3 SW27 linear 10% to 30% sucrose gradients. ϕ X DNA-containing fractions were collected and further separated and analyzed. If necessary, ϕ X DNA was concentrated by precipitation with isopropanol.

(b) Centrifugation procedures

The centrifugation procedures, the neutral high-salt sucrose gradients and PrI_2/CsCl gradients were performed as described previously (Baas *et al.*, 1976).

(c) Restriction enzyme analysis

The incubation of ϕ X174 replicative intermediates with the restriction endonuclease *Pst*I from *Providentia stuartii* 164 (Smith *et al.*, 1976) were performed as described previously (Baas *et al.*, 1978).

(d) Electron microscopy

Replicative intermediates of ϕ X174 DNA were spread for electron microscopy by the formamide modifications of the protein monolayer technique (Davis *et al.*, 1971). The conditions used were 0.1 to 0.5 μ g DNA/ml in 50% formamide, 0.1 M-ammonium acetate, 10 mM-EDTA (pH 8.0), 0.02% cytochrome *c* (extracted from horse heart), spread onto a hypophase of triple distilled water (Keegstra *et al.*, 1977). Replicative intermediates cleaved with the restriction endonuclease *Pst*I were spread for electron microscopy without further purification of the DNA/enzyme mixture. The specimens were examined with a Philips EM301 and photographed on Kodalith LR film. The magnification was calibrated on each film using a carbon grating replica (Fullam, 2160 lines/mm). Length measurements were performed with a Hewlett Packard 9864A digitizer connected to a 9820A calculator.

3. Results

(a) Isolation of ϕ X174 replicative intermediates

ϕ X174 infected *E. coli* H502 was pulse-labeled during the period of ϕ XRF DNA replication and the cell lysate was centrifuged through a neutral sucrose gradient, as described in Materials and Methods. Fractions containing pulse-labeled DNA were

pooled and then subjected to PrI_2/CsCl gradient centrifugation as described by Baas *et al.* (1978). Pulse-labeled DNA bands: (1) at the position heavier than the RFI marker; (2) a considerable amount bands in between the RFI and RFII markers; and (3) pulse-labeled DNA coincides with the RFII marker. The DNA at these three positions was collected separately and analyzed in the electron microscope. Most of the information was derived by analysis of the DNA from the fractions in between the RFI and RFII markers (intermediate fraction), in which the ratio of replicative intermediates to mature RFI and RFII molecules is relatively high.

(b) *Classification of DNA molecules*

The contents of the intermediate fraction were analyzed by scanning about 600 molecules in the electron microscope. A total of 287 (44.7%) circular DNA molecules of ϕX174 genome length were observed, 74 (11.5%) linear DNA molecules of genome length, 262 (40.8%) linear DNA molecules smaller than genome length, 19 (3.0%) rolling circles and two connected DNA circles (< 0.5%). The 287 circular DNA molecules are subdivided in 49 single-stranded, 51 partially duplex, 80 RFI and 107 RFII DNA molecules. It should be realized that the detection limit of the present technique is a DNA fragment with a minimum length of 50 base-pairs. Hence single-stranded DNA molecules containing small double-stranded DNA regions will also be classified as single-stranded DNA, and double-stranded DNA molecules with small single-stranded DNA gaps will be classified as double-stranded DNA. The results indicate that at least 18.5% of the molecules in the intermediate fraction including single-stranded circular ϕX174 DNA, partially duplex ϕX DNA and rolling circles are replicative intermediates. Further electron microscopic analysis of the three fractions of the PrI_2/CsCl gradient was performed by scanning only those molecules that were further analyzed, namely rolling circles, partially duplex DNA circles and structures consisting of two connected DNA circles. Single-stranded DNA circles were not included in this analysis. Table 1 presents a classification of the different molecules.

(c) *Rolling circle intermediates*

Rolling circles were observed in the intermediate and heavy fractions of PrI_2/CsCl gradients. The various types of rolling circle DNA molecules were divided into three classes (Table 1).

TABLE 1
Classification of ϕX174 replicative intermediates

	Rolling circles						
	One tail		Two tails at one point		Tails at different points		
	ss	Partially ds	ss	Partially ds	2	3	4 tails
No. of molecules	30	10	46	5	9	2	1
Partially duplex DNA circles							
Double-stranded regions			1	2	3	4	
No. of molecules			160	13	9	2	
Two connected DNA circles							
		ds-ds		ds-partially ds		ds-ss	
No. of molecules		29†		6		5	

† In 3 of these molecules the 2 double-stranded (ds) DNA circles were connected to each other by a piece of DNA. ss, single-stranded.

FIG. 1. Examples of ϕ X174 rolling circles. (a) Rolling circle with a single-stranded DNA tail; (b) rolling circle with a partially duplex DNA tail; (c) rolling circle with 2 single-stranded DNA tails protruding from 1 point (partial branch migration); (d) rolling circle with 2 DNA tails protruding from 1 point (partial branch migration); 1 of the tails is partially duplex DNA. The bar indicates 0.2 μ m.

(i) Rolling circles with only one tail; the tail in these molecules was either single-stranded or partially double-stranded DNA. Examples of these molecules are presented in Figure 1(a) and (b), respectively. The molecules confirm the classical rolling circle model (Gilbert & Dressler, 1968) of ϕ X174 RF DNA replication. Replication is initiated by the introduction of a nick in the viral DNA strand. The old viral DNA strand is then elongated starting at the 3'-OH end displacing the 5' end. The complementary DNA strand remains circular and serves as a template. The tail of the rolling circle is either single-stranded or partially double-stranded DNA. This suggests

FIG. 2. Schematic diagram of branch migration. (a) Branch migration in a rolling circle; (b), (c), (d) and (e) listing of the possible structures which one can expect after branch migration before and/or after *Pst*I cleavage. Classes (b), (c) and (d) are subdivided into rolling circles in which DNA replication has not reached the *Pst*I cleavage site, and molecules in which DNA replication has proceeded beyond the *Pst*I cleavage site. (~~~~) Newly synthesized viral DNA strand; O, origin of viral DNA strand replication; P, *Pst*I cleavage site.

that the synthesis of the complementary DNA strand can, but does not necessarily, start when a startpoint of the complementary strand DNA synthesis is exposed.

(ii) Rolling circles with two DNA tails protruding from the same site of the duplex DNA circle. One of the tails is always single-stranded and the other tail is either single or (partially) double-stranded DNA (Fig. 1(c) and (d)). These molecules arise from the rolling circle by branch migration (Lee *et al.*, 1970; Francke & Ray, 1972), as shown in Figure 2(a). Due to branch migration the newly synthesized DNA strand is (partially) displaced from the DNA circle by the old viral DNA strand. This yields DNA molecules with two tails in which the newly synthesized displaced tail is always single-stranded DNA and the other tail (the old viral strand with the 5' end) is either (partially) double or single-stranded DNA depending on whether complementary strand DNA synthesis has started or not.

(iii) Rolling circles with more than one DNA tail protruding from different sites of the double-stranded DNA circle (Fig. 8 (a)). These structures could arise by multiple starts of the viral strand DNA synthesis. Since there is a specific origin of viral strand synthesis (Baas *et al.*, 1976; Langeveld *et al.*, 1978) the length of the tails should be such, that the ends of the tails come together at the same position on the double-stranded DNA circle after folding them back in the same direction. About half of the molecules found in this analysis meet these requirements.

(d) *Rolling circles cleaved with the restriction endonuclease PstI*

It has been shown by experiments *in vivo* (Baas *et al.*, 1976) as well as *in vitro* (Eisenberg *et al.*, 1977) that the origin of RF DNA replication is located at a unique site of the ϕ X174 genome. This site has been located at nucleotide 4305 of the complete sequence of ϕ X174 DNA (Sanger *et al.*, 1978) by Langeveld *et al.* (1978), which position corresponds to a distance of 1081 nucleotides (20.1 map units; 1 map unit corresponds to 1% of the total length of the genome) from the single *PstI* cleavage site. ϕ X RF DNA replication is unidirectional from the origin in the direction of the genes *A*, *B* . . . *H* (Baas & Jansz, 1972), i.e. in the order of the numbering of the ϕ X DNA sequence (Sanger *et al.*, 1978).

In order to determine whether the tails of the rolling circles as studied in this work originate from the same position, the intermediate and heavy fractions were treated with *PstI* and the linear tailed DNA molecules obtained were analyzed in the electron microscope. This analysis includes four different types of linear branched DNA molecules (Table 2).

(i) Molecules with one tail shorter than 20 map units, in which the branchpoint of the tail is located between 0 and 20 map units from the *PstI* cleavage site (Fig. 3(a)). In order to determine the startpoint of tail formation the tail must be folded back away from the *PstI* cleavage site (Fig. 2(b) 1). Molecules with one tail longer than 20 map units (Fig. 3(b)). The tails of these molecules must be folded back over the *PstI* cleavage site to determine the origin of tail formation, as shown in Figure 2(b) 2. The analysis of 25 molecules shows that tail formation starts at 22.1 ± 3.9 map units from the *PstI* cleavage site (Table 2).

(ii) Molecules with a tail of variable length in which the branchpoint of the tail is located at approximately 20 map units from the *PstI* cleavage site. This category corresponds to *PstI*-cleaved rolling circles after complete branch migration of the displaced tail (Fig. 2(c)). Examples of these molecules are shown in Figure 3(c) and (d).

FIG. 3. Examples of ϕ X174 rolling circles treated with the restriction endonuclease *Pst*I. (a) DNA molecule with a short single-stranded tail, the branchpoint of the tail is located between 0 and 20 map units from the *Pst*I cleavage site; (b) DNA molecule with a long single-stranded DNA tail, the branchpoint of the tail is located more than 20 map units from the *Pst*I cleavage site; (c) DNA molecule with a short single-stranded DNA tail which is located at the startpoint of rolling circle replication (complete branch migration); (d) DNA molecule with a long single-stranded DNA tail which is located at the startpoint of rolling circle replication (complete branch migration). The bar indicates 0.2 μ m.

TABLE 2

Rolling circles cleaved with the restriction endonuclease PstI

	Location of the origin of tail formation in map units
No branch migration (Fig. 2(b))	22.1 ± 3.9 (25)
Complete branch migration (Fig. 2(c))	21.0 ± 3.7 (30)
Partial branch migration (Fig. 2(d))	21.1 ± 3.5 (46)
Branch migration before and after <i>PstI</i> cleavage (Fig. 2(e))	not determined

The figures in the Table refer to the analysis of 101 *PstI*-cleaved rolling circles. The figures are expressed in map units from the *PstI* cleavage site. One map unit is defined as 1% of the total length of the genome. The figures in parentheses refer to the number of molecules analyzed. Apart from this analysis 12 *PstI*-cleaved rolling circles give different values, larger than 26 or smaller than 16 map units, for the origin of tail formation; these molecules were left out. Rolling circles with a (partially) duplex DNA tail covering the *PstI* cleavage site, are cleaved by *PstI* in the circular as well as in the linear part of the molecule yielding Y-shaped structures. Eight of these molecules were found, but they provided no information for this analysis.

The analysis of 30 of these molecules shows that the branchpoint of the tail is located at 21.0 ± 3.7 map units from the *PstI* cleavage site (Table 2).

(iii) Molecules with two tails protruding from one point of the molecule; these molecules arise by partial branch migration as shown in Figure 2(d). Examples of these molecules are shown in Figure 4 (Fig. 4(a) corresponds to Fig. 2(d) 1). If both tails are single-stranded DNA (Fig. 4(c)) we do not know which one of the two tails is the old viral DNA strand. Then the direction of folding away from the *PstI* cleavage site or back over the *PstI* cleavage site is unknown and in that case all possibilities were tested and the figures which come closest to a distance of 20 map units from the *PstI* site were taken. If a partially duplex DNA tail is present we know that this tail represents the old viral DNA strand. This tail must be folded back away from the *PstI* cleavage site if it is shorter than 20 map units (Fig. 2(d) 1 and 2). If the tail is longer than 20 map units the tail must be folded back over the *PstI* cleavage site in order to determine the origin of tail formation (Fig. 2(d) 3 and Fig. 4(b)). The analysis of 46 partially branch migrated *PstI*-cleaved rolling circles indicated a distance of 21.1 ± 3.5 map units from the *PstI* cleavage site for the startpoint of tail formation (Table 2).

(iv) Linear double-stranded DNA molecules of genome length with a single-stranded DNA tail of variable length at one end (Fig. 4(d)). These molecules arise from rolling circles in which replication has proceeded beyond the *PstI* cleavage site. After *PstI* cleavage a DNA fragment is completely removed by branch migration (Fig. 2(e)). As shown in Figure 2(e) 1 the length of the single-stranded DNA part of these molecules is unrelated to the position of the origin of tail formation. Although some of these molecules have a single-stranded DNA part of approximately 20 map units (Fig. 2(e) 2) they were not used in the present analysis.

(e) *Partially duplex DNA circles*

Partially duplex circular DNA molecules were found by electron microscopy in the intermediate and the RFII fraction of PrI_2/CsCl gradients. They represent 43% of all replicative intermediates in the intermediate fraction (see Results, section (b)).

FIG. 4. Examples of ϕ X174 rolling circles treated with the restriction endonuclease *Pst*I. (a) DNA molecule with 2 short single-stranded DNA tails (partial branch migration), the branch-point of the tails is located between 0 and 20 map units from the *Pst*I cleavage site; (b) DNA molecule with 2 tails protruding from the same point (partial branch migration), 1 of the tails is partially duplex DNA; (c) DNA molecule with 2 long single-stranded DNA tails protruding from the same point (partial branch migration); (d) linear double-stranded DNA molecule of ϕ X genome length with a single-stranded DNA tail at 1 end. The bar indicates 0.2 μ m.

FIG. 5. Examples of ϕ X174 partially duplex DNA molecules during ϕ X RF DNA replication before and after cleavage with the restriction enzyme *Pst*I. (a) Partially duplex DNA circle with 1 DNA fragment; (b) partially duplex DNA circle with 3 DNA fragments; (c) partially duplex DNA molecule with 1 DNA fragment after cleavage with *Pst*I (the *Pst*I cleavage site is in the duplex DNA part); (d) partially duplex DNA molecule with 2 DNA fragments after cleavage with *Pst*I, 1 of the DNA fragments covered the *Pst*I cleavage site. The bar indicates 0.2 μ m.

As shown earlier (Baas *et al.*, 1978) the partially duplex DNA circles are formed as one of the products of rolling circle DNA replication. They represent circular viral DNA strands containing fragments of the complementary DNA strand. Most of the molecules contain one fragment (Fig. 5(a)), although about 25% of the molecules (Table 1) have more than one fragment (Fig. 5(b)), up to four fragments per ϕ X174 single-stranded DNA circle. The presence of partially duplex DNA molecules 20 minutes after infection suggests that the observed molecules are intermediates in the synthesis of the complementary DNA strand during ϕ X RF DNA replication and not intermediates in the synthesis of the parental ϕ X RF DNA. The appearance of partially duplex DNA circles with more than one DNA fragment in a significant amount indicates that the synthesis of the complementary DNA strand during ϕ X RF DNA replication does not start at a unique site and occurs in a discontinuous fashion. This observation is in agreement with the results of others (Schröder *et al.*, 1973; Eisenberg & Denhardt, 1974*a,b*; Eisenberg *et al.*, 1975).

(f) *Partially duplex DNA circles cleaved with the restriction endonuclease PstI*

If there are specific startpoints for the synthesis of the complementary DNA strand one would expect that one of the single-strand to double-strand DNA junctions of a particular fragment in partially duplex DNA circles should be at a specific site on the ϕ X genome. Assuming that there are no specific termination sites the other single-strand to double-strand DNA junction of this fragment can be found at any location on the ϕ X genome. In order to determine the assumed startpoints, preparations containing partially duplex DNA circles were digested with the restriction endonuclease *PstI* (Fig. 5(c) and (d)). In the resulting linear partially duplex DNA molecules the distances of the single to double-strand DNA junctions from the *PstI* cleavage site were measured and used to make a histogram relating the measured lengths to the number of molecules (not shown). This histogram shows six peaks of dominating length-classes suggesting that there are six preferential sites on the ϕ X genome where the single to double-strand DNA junctions are located. The linear partially duplex DNA molecules were then aligned to obtain the best fit of their single to double-strand DNA junctions with these six sites (Fig. 2). The results indicate that in 97% of the molecules at least one single to double-strand DNA junction can be placed at either one of the six fixed locations on the ϕ X genome.

TABLE 3
*Initiation sites of complementary DNA strand synthesis during
 ϕ X174 RF DNA replication*

Initiation site	No. of starts
13.3 $\pm$ 1.7	7
27.4 $\pm$ 4.4	16
53.3 $\pm$ 1.9	19
64.3 $\pm$ 3.6	38
79.3 $\pm$ 3.4	23
91.9 $\pm$ 2.1	14

The locations of the initiation sites are given in map units from the *PstI* cleavage site (nucleotide 5386 in the ϕ X DNA sequence given by Sanger *et al.*, 1978).

FIG. 6. Representation of *Pst*I-cleaved ϕ X174 partially duplex DNA molecules. The lengths of the molecules were normalized to 1 and compiled in this Figure. Each bar refers to a double-stranded region in the DNA molecule. The molecules were orientated in such a way that the best fit to the 6 initiation sites of the complementary DNA strand synthesis was obtained. The initiation sites are indicated by vertical lines with arrows. The direction of replication for the complementary DNA strand is from left to right. Therefore the left end of the molecules corresponds to nucleotide 5386 in the ϕ X174 DNA sequence of Sanger *et al.* (1978). On top of the Figure the reversed genetic map of ϕ X174 is shown.

FIG. 7. Examples of ϕ X174 DNA "termination structures". (a) Two double-stranded DNA circles connected to each other at 1 point; (b) partially duplex DNA circle connected to a double-stranded DNA circle; (c) single-stranded DNA circle connected to a double-stranded DNA circle; (d) double-stranded-double-stranded DNA structure after cleavage with the restriction endonuclease *Pst*I; (e) double-stranded-partially duplex DNA termination structure after *Pst*I cleavage; (f) double-stranded-single-stranded DNA termination structure after *Pst*I treatment. The bar indicates 0.2 μ m.

We suggest that these six sites represent initiation sites for the synthesis of the complementary DNA strand. The direction of synthesis of the complementary DNA strand is counterclockwise in the direction of the genes *A-H-G-F . . . A*, as indicated in Figure 6. Table 3 gives the position of the initiation sites and the number of molecules with a start of complementary DNA strand synthesis at that particular site.

(g) *Termination structures*

In specimens made from the intermediate or the combined RFI and heavy fractions of PrI_2/CsCl gradients, molecules were found that consist of two ϕ X174 genome length DNA rings which are connected to each other. There are four types of these molecules (Table 1).

(i) Two double-stranded DNA circles connected to each other at only one point (Fig. 7(a)); these molecules are indistinguishable from catenanes and recombination intermediates (figure-8 molecules) as found by others (Gordon *et al.*, 1970; Thompson *et al.*, 1975; Benbow *et al.*, 1975).

(ii) One completely double-stranded DNA circle connected to a partially duplex DNA circle at one point (Fig. 7(b)). These molecules cannot be recombination intermediates. We consider two other possibilities, (a) the partially duplex DNA circle is concatenated with an RF molecule; and (b) it is a replicative intermediate involved in the termination phase of the rolling circle DNA replication (called termination structure).

(iii) One completely double-stranded DNA circle with a completely single-stranded DNA circle attached to it at one point (Fig. 7(c)). For this category of molecules the same explanations are possible as mentioned for type (ii).

(iv) Two double-stranded DNA circles connected to each other by a short stretch of linear DNA (Fig. 8(d)). We observed that the maximum length of the connecting

TABLE 4

Location of the point of contact after PstI cleavage in structures in which two DNA circles were connected to each other at one point

ds-ds DNA structures		ds-partially ds DNA	
Recombination structures	Termination structures	Termination structures	ds-ss DNA† Termination structures
37.2-34.6	16.6-15.4	21.9-22.1	19.7
28.8-26.6	25.3-28.7	26.2-26.0	24.1
41.9-40.5	28.9-24.9	29.2-27.3	23.3
46.5-49.2	22.0-19.0	22.4-20.2	17.4
45.5-48.8	26.3-23.9	23.9-20.7	25.5
44.9-47.3	21.2-22.2		
12.0-12.1			
30.1-29.3			
45.2-47.5			
Mean value	22.9 $\pm$ 4.3 (12)	24.0 $\pm$ 3.0 (10)	22.0 $\pm$ 3.4 (5)

† *PstI* does not cleave single-stranded DNA (Blakesley *et al.*, 1977), therefore only 1 figure per molecule is available.

The distances of the points of contact to the *PstI* cleavage site are expressed in map units. The matched data represent the values of the 2 molecules present in a recombination or a termination structure. The numbers of values used for the determination of the mean value are given in parentheses. ds, double-stranded; ss, single-stranded.

FIG. 8. Examples of ϕ X174 DNA molecules occurring at low frequencies during ϕ X RF DNA replication. (a) Rolling circle with 3 (partially branch migrated) DNA tails; (b) rolling circle with 2 single-stranded DNA tails protruding from 1 point (partial branch migration), the tail is longer than 1 genome length; (c) rolling circle with 2 tails protruding from 1 point (partial branch migration), 1 of the tails is partially duplex DNA and much longer than ϕ X genome length; (d) two double-stranded DNA circles connected by a single-stranded DNA piece; (e) a looped DNA molecule. The bar indicates $0.5 \mu\text{m}$.

single-stranded DNA was slightly smaller than one ϕ X genome length. In some cases (partially) duplex DNA was seen as the connecting DNA chain. These structures are most likely the result of defective termination of ϕ X RF DNA replication.

(h) *Termination structures cleaved with the restriction enzyme PstI*

To distinguish between termination structures and figure-8 molecules or catenanes in an unambiguous way, the specimens were studied after cleavage with the restriction endonuclease *PstI*. All catenated structures will disappear after cleavage of one of the two rings with *PstI*. After cleavage with *PstI* three types of chi-shaped structures were found in the specimens (Table 4).

(i) A linear double-stranded DNA molecule attached to another linear double-stranded DNA molecule (Fig. 7(d)) with two pairs of equal-length arms. In six of these molecules the branchpoint is at 22.9 ± 4.3 map units from the *PstI* cleavage site (Table 4). Nine molecules are found with the branchpoint at another place on the molecule, indicating that these molecules are recombination intermediates (Potter & Dressler, 1976).

FIG. 9. Model for ϕ X174 RF DNA replication *in vivo*. ϕ X RF DNA replication starts by the introduction of a nick made by the gene A protein in the viral DNA strand (v) of supercoiled RFI DNA. This nick creates a 3'-OH-bounded G residue at nucleotide number 4305 (Langeveld *et al.*, 1978) of the ϕ X DNA sequence (Sanger *et al.*, 1978). After nicking gene A protein ($\circ$) remains bound at the 5' end of the nick (Ikeda *et al.*, 1976; Eisenberg *et al.*, 1977; Langeveld *et al.*, 1978) (I). Viral DNA strand synthesis starts at the origin according to the rolling circle mechanism (Gilbert & Dressler, 1968) (II). Discontinuous synthesis of new complementary DNA strands (c) can occur on the displaced viral DNA strand tail (IIB). During replication the 5' end of the rolling circle tail may be held back to the growing point by virtue of a non-covalent interaction between the proteins on the tail and the replication enzyme complex, as suggested by Eisenberg *et al.* (1977). Termination of a round of rolling circle DNA replication takes place by circularization of the genome-length tail (III) and separation (IV) of the daughter DNA molecules, resulting in one RFI DNA molecule containing a specific gap in the viral DNA strand, which continues to replicate (V) and a single-stranded DNA viral ring (IVB) or an RF DNA molecule with one or multiple gaps in the complementary DNA strand (IVB), which is converted (VI, VII, VIII) *via* relaxed RFI into supercoiled RFI DNA.

(ii) A linear double-stranded DNA molecule connected to a partially duplex linear DNA molecule (Fig. 7(e)). The branchpoint in these molecules (a total of five molecules was found) is located at 24.0 ± 3.0 map units from the *Pst*I cleavage site (Table 4).

(iii) A linear double-stranded DNA molecule connected to a single-stranded DNA circle (Fig. 7(f)). The branchpoint in these molecules (a total of five molecules was found) is located at 22.0 ± 3.4 map units from the *Pst*I cleavage site (Table 4).

(i) Other structures

(i) Rolling circles with a single-stranded or (partially) duplex DNA tail longer than ϕ X174 genome length were seen occasionally (Fig. 8(b) and (c)). In these molecules the DNA synthesis has not stopped, but proceeded (several times) beyond the termination site.

(ii) One out of 5000 observed molecules in a curious looped structure as shown in Figure 8(e).

4. Discussion

Biochemical studies (Baas *et al.*, 1978) in combination with the electron microscopic analysis of replicative intermediates, described in this paper, confirm and extend the classical rolling circle model of ϕ X174 RF DNA replication (Gilbert & Dressler, 1968). A scheme which summarizes our observations is presented in Figure 9. It should be noticed that the intention of this paper is not to present a quantitative description of all ϕ X RF DNA replicative intermediates within the cell. During our studies (Baas *et al.*, 1978, and unpublished data) we have observed that the yield of the various DNA replicative intermediates depends on the method used to stop the pulse-labeling as well as on the method used to isolate the DNA replicative intermediates. In this study the pulse-labeling was stopped by fast cooling in the presence of KCN and NaN₃, and ϕ X DNA replicative intermediates were isolated in a lengthy series of steps. A selective loss of a particular DNA form through these many steps cannot be excluded. In a first screening we observed three different types of ϕ X DNA replicative intermediates, rolling circles, partially duplex DNA circles and termination structures. The results described in this paper are from a detailed electron microscopic study of these three types of molecules, which were obtained from different experiments.

Our results indicate that the startpoint of rolling circle DNA replication is located at a unique site on the ϕ X genome, which is located at 21.3 ± 3.7 map units from the *Pst*I cleavage site (Table 2). Koths & Dressler (1978) have found that the startpoint of the rolling circle DNA replication during progeny single-stranded DNA synthesis is located at the same place. A position of 21.3 map units also corresponds to the position of the replication origin of ϕ X RF DNA replication as determined biochemically by studies *in vivo* (Baas *et al.*, 1976) and *in vitro* (Ikeda *et al.*, 1976; Eisenberg *et al.*, 1977; Langeveld *et al.*, 1978). In agreement with the results of Koths & Dressler (1978) we find that the majority of the rolling circles (85%) have a single-stranded DNA tail although a greater percentage of the tails had a duplex region in our experiments. This indicates that the synthesis of the complementary strand can already start at the single-stranded DNA tail of the rolling circle (Fig. 9(B)). A round of rolling circle DNA replication yields two daughter DNA molecules one of which is double-stranded and continues to replicate (Fig. 9(V)), whereas the other is

either single-stranded or partially double-stranded (Fig. 9(IV)). The latter structures are converted (VI, VII, VIII) *via* relaxed RFI (Baas *et al.*, 1978) into superhelical RFI.

Eisenberg *et al.* (1975) have shown that the multiple gaps in the complementary DNA strand of partially duplex DNA circles are distributed in a non-random fashion along the ϕ X genome. Our electron microscopic analysis of the partially duplex DNA rings after *Pst*I cleavage supports that conclusion, and indicates that complementary DNA strand synthesis does not start at random positions on the ϕ X genome. At least six initiation sites for the synthesis of the complementary DNA strand could be located (Table 3 and Fig. 6). It should be noted that our analysis does not include partially duplex DNA circles with a single-stranded region at the *Pst*I cleavage site which are therefore not cleaved by the *Pst*I enzyme (Blakesley *et al.*, 1977). Therefore the start frequencies at the sites closest to the *Pst*I cleavage site are probably over-estimated. Also we cannot exclude the possibility that there are additional initiation sites. The most frequently used initiation site at 64.3 map units from the *Pst*I cleavage site could be subdivided into two initiation sites at 61 and 69 map units, respectively. There is also no evidence for the initiation of the ϕ X parental complementary strand synthesis at a single defined site from studies *in vivo* (Hess *et al.*, 1973; Baas *et al.*, 1978; Hourcade & Dressler, 1978) as well as *in vitro* (McMacken *et al.*, 1977). However, there may be a preferred initiation site near the gene *A-H* boundary and two or three other sites in genes *F*, *G* and *H* (Hourcade & Dressler, 1978; Zuccarelli *et al.*, 1976).

As indicated in Figure 9(III) we assume that the tail of the rolling circle undergoes circularization before the two daughter DNA molecules come apart. The evidence for this assumption is based on the occurrence of structures consisting of two genome-length DNA rings connected at one point. In our studies we identified several structures which consisted of a double-stranded DNA circle connected to a single-stranded, a partially duplex or a double-stranded DNA circle. In the structures in which a double-stranded DNA circle is connected to a single-stranded or a partially duplex DNA circle the points of contact are 22 and 24 map units from the *Pst*I cleavage site, respectively.

The molecules in which two double-stranded DNA circles are connected to each other at one point are indistinguishable from recombination structures. However, the distribution of the points of contact in the recombination structures should be uniform along the genome because branch migration causes a random movement of the points of contact (Lee *et al.*, 1970; Potter & Dressler, 1976; Thompson *et al.*, 1978). Our data of the *Pst*I-treated specimens show a distinct peak at the region between 15 and 25 map units from the *Pst*I cleavage site. This suggests that part of the molecules in which two duplex DNA rings are connected do not belong to the recombination structures, but are termination structures.

No looped rolling circles with a tail smaller than one genome length were found in our preparations. However, it is possible that the end of the tail of the rolling circle is loosely associated *via* a protein with the origin (Knippers *et al.*, 1969) or with the replication fork (Eisenberg *et al.*, 1977) during ϕ X RF DNA replication *in vivo* and that this linkage is broken during our isolation procedure or electron microscopic preparation technique. Our isolation procedure includes treatment with sodium dodecyl sulphate/Pronase and extraction with phenol and the molecules were spread for electron microscopy without crosslinking. The looped rolling circles observed in the electron microscope by Eisenberg *et al.* (1977) during ϕ X DNA replication *in*

vitro were non-deproteinized and crosslinked with glutaraldehyde before spreading. For the visualization of looped rolling circles crosslinking was necessary. When crosslinking was omitted rolling circles with free ends were observed. Therefore, if looped rolling circles exist during ϕ X RF DNA replication *in vivo*, they are probably disrupted in our experiments. The proposed non-covalent protein linkage at the 5' end of the tail with the template ring must, therefore, be less stable than the linkage in the termination structures. Our data suggest that during termination of a round of rolling circle replication the 5' end of the tail becomes stably associated with the origin region, i.e. when the origin of the displaced strand is exposed in a single-stranded DNA form. An attractive hypothesis for which there is no evidence, is that the association is caused by a covalent gene A protein-DNA linkage. The gene A protein, covalently bound at the 5' end of the tail (Ikeda *et al.*, 1976; Eisenberg *et al.*, 1977; Langeveld *et al.*, 1978) nicks the displaced strand at the origin and then joins the 3' and 5' ends of the displaced strand (Eisenberg *et al.*, 1977). This would terminate or interrupt viral DNA strand replication and yield circular viral DNA strands of genome length. The proposed termination structures may be intermediates in the process of circularization of the displaced viral strand.

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