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Bacteriophage ϕX174 and G4 RF DNA Replicative Intermediates

A Comparative Study Using Different Isolation Procedures

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We have isolated replicative intermediates of bacteriophage ϕX174 and the related bacteriophage G4, during RF (replicative form) DNA replication using different procedures. Biochemical and electron microscopic analysis of ϕX and G4 DNA replicative intermediates isolated by the same procedure, showed no significant differences. In the replication cycle of both phages rolling circles and gapped RF DNA molecules are the predominant replicative intermediates. It is concluded that G4 RF DNA also replicates according to a rolling circle model and not according to a D-looped replication model as proposed by Godson (1977*b*).

1. Introduction

Models for the DNA replication of various organisms have been proposed, based upon biochemical and electron microscopic analysis of their DNA replicative intermediates. For ϕX RF (replicative form) DNA replication a rolling circle type of DNA replication has been well documented (for a review see Baas & Jansz, 1978; for a model see Fig. 1). In several laboratories rolling circles during RF DNA replication have been observed (Knippers *et al.*, 1969*a*; Schröder & Kaerner, 1972; McFadden & Denhardt, 1975; Fukuda & Sinsheimer, 1976; Machida *et al.*, 1977; Baas *et al.*, 1978; Koths & Dressler, 1978; Keegstra *et al.*, 1979). However, for the related bacteriophage G4 no rolling circles could be detected during RF DNA replication and a D-looped replication model has been proposed (Godson, 1977*b*). The reason for this difference may be the different method that was used to isolate the G4 DNA replicative intermediates. During our studies on the mechanism of bacteriophage ϕX174 RF DNA replication (Baas *et al.*, 1978; Baas & Jansz, 1978; Keegstra *et al.*, 1979) we have observed that the yield of the various DNA replicative intermediates strongly depends on the method used to stop the pulse-labelling as well as on the method used for the isolation of the replicative intermediates. In experiments with ϕX using the isolation procedure described by Godson in his studies about the DNA replication of bacteriophage G4 (Godson, 1977*a*,*b*; Martin & Godson, 1977), we obtained a very low yield of rolling circles during ϕX RF DNA replication. Therefore we compare in this paper the

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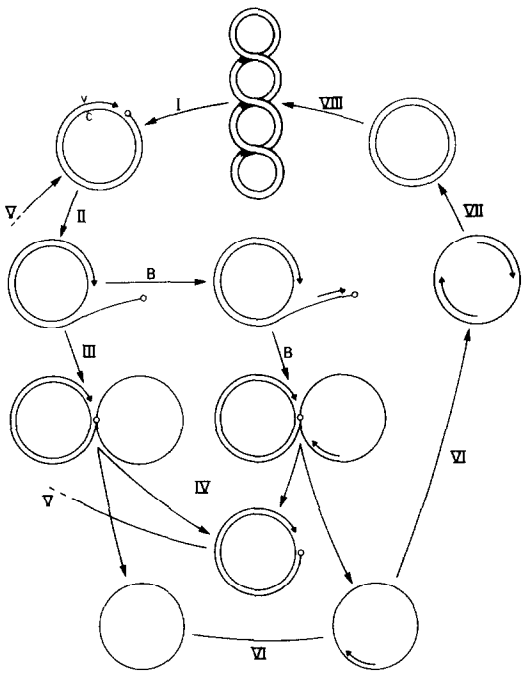

FIG. 1. Model for ϕX RF DNA replication *in vivo*. ϕX RF DNA replication starts by the introduction of a nick made by the cistron A protein in the viral strand of supertwisted RFI DNA. This nick creates a 3′ OH bounded G residue at nucleotide number 4305 (Langeveld *et al.*, 1978) of the ϕX DNA sequence (Sanger *et al.*, 1977,1978). After nicking, cistron A protein (○) remains bound at the 5′ end of the nick (Ikeda *et al.*, 1976; Eisenberg *et al.*, 1977; Langeveld *et al.*, 1978) (I). Viral strand synthesis starts at the origin according to the rolling circle mechanism (Gilbert & Dressler, 1968) (II). Discontinuous synthesis of new complementary strands can occur on the displaced viral strand tail (II,B). 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 circles replication takes place by circularisation of the genome length tail (III) and separation (IV) of the daughter molecules, resulting in one RFII DNA containing a specific gap in the viral strand, which continues to replicate (V), and a single-stranded viral ring (IV) or an RF molecule with one or multiple gaps in the complementary strand (IV,B), which is converted (VI, VII, VIII) *via* relaxed RFI into supertwisted RFI DNA.

replicative intermediates of ϕX and G4 RF DNA replication isolated using different procedures.

ϕX and G4-infected cells were pulse-labelled during RF DNA replication, and from the same cells ϕX and G4 DNA replicative intermediates were isolated using different isolation procedures. No significant differences were found between ϕX and G4 DNA replicative intermediates, when they were isolated, using the same procedure. The results indicate that also G4 RF DNA replicates as a rolling circle.

2. Materials and Methods

(a) *Phage and bacterial strains*

Escherichia coli strain C122 was used as the normal host for plating and propagation of ϕX and G4.

E. coli strain H502 (*thy*$^-$, *uvrA*, *endoI*$^-$, *su*$^-$) was used as the host in the experiments described.

(b) *Media and solutions*

E. coli H502 was grown in TPG medium (Levine & Sinsheimer, 1968) after addition of 200 μg Casamino acids (Difco)/ml and 2 μg thymine/ml. Tris/EDTA buffer is 0·05 M-Tris·HCl, 0·005 M-EDTA (pH 8·0). Borate/EDTA/NaN_3/KCN contains 0·05 M-tetrasodium borate, 0·0025 M-EDTA, 0·05 M-NaN_3, 0·005 M-KCN.

(c) *Centrifugation procedures*

In the Figures shown, sedimentation is always from right to left. The centrifugation procedures, neutral high salt sucrose gradients, alkaline sucrose gradients, propidium diiodide/CsCl gradients (100 μg PrI_2†/ml) and poly(U,G)/CsCl gradients for the separation of complementary and viral strands of ϕX and G4 RF DNA, after denaturation for 3 min at 100°C, were performed as described previously (Baas *et al.*, 1976).

(d) *Benzoyl-naphtoyl-DEAE-cellulose chromatography*

BND-cellulose (benzoyl-naphtoyl-DEAE-cellulose; Serva) chromatography was performed with small BND-cellulose columns of 0·5 cm × 0·5 cm. DNA in 0·3 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA (pH 8·1) was bound completely to these columns. Subsequent elution with 1 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA released double-stranded DNA (double-stranded fraction), while further elution with 1 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA, 2% (w/v) caffein (caffein fraction) yielded the single-stranded DNA-containing molecules (Kiger & Sinsheimer, 1969).

(e) *Infection and labelling of the cells*

E. coli H502 was grown at 37°C in 1600 ml of TPG medium, containing 200 μg Casamino acids/ml and 2 μg thymine/ml. At 4×10^8 cells/ml the cells were collected by centrifugation and resuspended in 120 ml fresh medium. In order to suppress bacterial DNA synthesis, the cells were treated with 50 μg mitomycin C/ml (Sigma Chemical Corp.) for 10 min at 37°C (Lindqvist & Sinsheimer, 1967). To remove mitomycin C the cells were pelleted again and suspended in 1500 ml prewarmed (37°C) TPG medium. The concentration of thymine in the TPG medium was lowered to 0·5 μg/ml in order to enhance the specific activity of the label. Then the culture was divided in 2 portions. To each half 16 ml of chloramphenicol (2 mg/ml) was added and 5 min later 1 culture was infected with ϕX, suspended in 25 ml TPG medium, with a multiplicity of infection of 5 and the other culture was infected in the same way with G4. Then 20 min after infection the cultures were pulse-labelled during 15 s with 4 mCi [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol; Radiochemical Centre, Amersham, U.K.) dissolved in 50 ml TPG medium. The pulse label was terminated by pouring the infected cultures at room temperature into a stainless steel beaker, containing 600 ml ethanol, 164 ml water, 19 ml phenol, 13 ml 0·25 M-EDTA, 3·35 ml 3 M-sodium acetate (pH 5·5) (Manor *et al.*, 1969). Thereafter the cells were collected by centrifugation and washed twice with 120 ml borate/EDTA/NaN_3/KCN in the cold. Then the ϕX and G4-infected cells were again divided into halves. From one portion DNA replicative intermediates were isolated according to method I and from the other half according to method II.

(f) *Isolation procedures of DNA replicative intermediates*

(i) *Isolation method I*

A 200-ml portion of the culture was resuspended at 0°C in a Sorvall SS34 centrifuge tube in 16 ml of 10% (w/v) sucrose, 0·05 M-Tris·HCl (pH 8·0). Then 2 ml of lysozyme (10 mg/ml in 0·25 M-Tris·HCl, pH 8·0) and 8 ml of 0·25 M-EDTA (pH 8·0) were added

† Abbreviations used: PrI_2, propidium diiodide; RFII, replicative form DNA with one or more discontinuities in either strand; RFI, replicative form DNA with both strands closed.

to the cells. After 30 min at 0°C 0·65 ml of Pronase (Calbiochem, B grade; 10 mg/ml in 0·05 M-Tris·HCl, pH 8·0, autodigested for 2 h at 37°C) and 2·0 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 water bath at 37°C and after 10 min the clear viscous lysate was placed in the cold room without cooling (4°C). After 14 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 dialysed against Tris/EDTA buffer and extracted twice with freshly distilled phenol. The phenol contained 0·1% (w/v) hydroxyquinoline and was equilibrated with Tris/EDTA buffer. Nucleic acids were precipitated by the addition of 0·1 vol. of 3 M-sodium acetate (pH 5·5) and 2 vol. of 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 μg RNAase/ml (Worthington Biochemical Corp.; pre-incubated for 10 min at 80°C) for 10 min at 37°C and layered directly on top of a linear 10% to 30% sucrose gradient.

(ii) *Isolation method II*

A 200-ml portion of the culture was resuspended in a Beckman polycarbonate centrifuge tube (type 40) at 0°C in 3·2 ml of 10% (w/v) sucrose, 0·05 M-Tris·HCl (pH 8·0). Then 0·32 ml of lysozyme (10 mg/ml in 0·25 M-Tris·HCl, pH 8·0) and 0·16 ml of a 0·25 M-EDTA solution (pH 8·0) were added to the cells. After 20 min at 0°C, 0·12 ml of a 5 M-NaCl and 0·2 ml of a 10% (v/v) Sarkosyl solution (Geigy) were added to the cells. Then the centrifuge tube was placed at room temperature for 30 min. Thereafter *E. coli* DNA was spun down at 5°C in a Beckman 50 Ti rotor for 1 h at 38,000 revs/min. Then, after dialysis of the supernatant solution against Tris/EDTA buffer, a Pronase treatment (final concn of Pronase 250 μg/ml) was given for 30 min at 37°C. Then the supernatant solution was extracted twice with freshly distilled phenol. Finally the replicative intermediates were isolated in a neutral high salt sucrose gradient after isopropanol precipitation and RNAase treatment (see above).

(g) *Electron microscopy*

DNA replicative intermediates 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 μg DNA/ml in 50% (v/v) formamide, 0·1 M-ammonium acetate, 10 mM-EDTA (pH 8·0), 0·01% (w/v) cytochrome *c* (extracted from horse heart), spread on to a hypophase of triple distilled water (Keegstra *et al.*, 1977). 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 digitiser connected to a 9820A calculator.

(h) *Preparation of uniformly labelled* [^{14}C]*G4 DNA and* [^{14}C]*G4 RF DNA*

Uniformly labelled [^{14}C]G4 viral strand DNA and [^{14}C]G4 RF DNA were prepared using the same procedures as described for φX (Baas *et al.*, 1978).

(i) *Measurements of radioactivity*

Samples from gradients and columns were diluted to 0·6 ml with water and mixed with 10 ml of a mixture consisting of 23 parts of toluene solution (5 g PPO and 0·05 g POPOP/l) and 7 parts Triton X100 (Rohm and Haas, Philadelphia).

3. Results

(a) *Preliminary observations and outline of the experiment*

For a biochemical and/or electron microscopic study of DNA replicative intermediates it is desirable to separate first the replicative intermediates into distinct

classes. For ϕX DNA replicative intermediates this separation has been achieved by PrI_2/CsCl gradient centrifugation, sometimes followed by BND-cellulose chromatography (Baas *et al.*, 1978). The position of DNA in PrI_2/CsCl gradients depends both on the conformation of the DNA (nicked, supercoiled or relaxed DNA) and on the proportion of single to double-stranded DNA in one molecule. BND-cellulose chromatography separates double-stranded DNA from DNA with single-stranded regions. Our biochemical (Baas *et al.*, 1978) and electron microscopic analyses (Keegstra *et al.*, 1979) of the different fractions of ϕX DNA replicative intermediates obtained after PrI_2/CsCl gradient centrifugation have shown, that the heavy fraction (see for an example Fig. 3(a), fraction H) consists mainly of rolling circles. In the intermediate fraction single-stranded DNA, rolling circles and gapped molecules were found and the RFII fraction contained besides RFII DNA, rolling circles with small tails and gapped molecules with short single-stranded regions. Especially for the biochemical detection of rolling circles, e.g. the detection of ^{3}H-labelled viral strand DNA longer than unit length, PrI_2/CsCl gradient centrifugation is an excellent separation procedure, because 90% of the label found in the heavy fraction after a short pulse consists of rolling circles. Preliminary experiments with ϕX using the isolation method described by Godson (1977*a*) showed a large reduction of the percentage of rolling circles. Therefore we decided to analyse ϕX and G4 DNA replicative intermediates, isolated by two different procedures from the same infected culture. Isolation method I is our standard procedure (Baas *et al.*, 1978) and isolation method II consists of the lysis procedure of Godson (1977*a*), followed by our purification method of the DNA replicative intermediates (see Materials and Methods). The purification of DNA replicative intermediates after centrifugation of the cell lysate was included in this method to obtain reproducible results. Direct separation of DNA replicative intermediates after centrifugation of the cell lysate in PrI_2/CsCl gradients resulted in difficulties with the spreading of the molecules for electron microscopy (W. Keegstra, unpublished results), and for some fractions bad separations of the complementary and viral strands in poly(U,G)/CsCl gradients were obtained. Furthermore, when the purification step was omitted, only 5% of the radioactivity of the heavy fraction was found again in the heavy fraction after rebanding in a second PrI_2/CsCl gradient; the remaining 95% was divided over the RFI, the intermediate and the RFII fraction.

The results described in the next sections were obtained from the following experiment. An *E. coli* H502 culture was split after mitomycin C treatment into two portions. One half of the culture was infected in the presence 40 μg chloramphenicol/ml with ϕX and the other half with G4. Twenty minutes after infection the cultures were pulse-labelled with [*methyl*-^{3}H]thymidine for 15 seconds. The pulse was stopped by pouring the infected cells into an ethanol/phenol mixture. Then the cultures were divided again into two halves. From one portion DNA replicative intermediates were isolated according to method I and from the other half according to method II.

(b) *Recovery of pulse-labelled DNA and fractionation of the DNA replicative intermediates*

Table 1 shows the amount of radioactivity present in the different supernatant fractions after centrifugation of the cell lysate. Three times more radioactivity was

TABLE I

Recovery of pulse-labelled φX and G4 DNA after centrifugation of the cell lysate

Organism	Lysis method	^{3}H radioactivity (cts/min)
φX	I	$3{\cdot}1 \times 10^6$
	II	$1{\cdot}0 \times 10^6$
G4	I	$1{\cdot}9 \times 10^6$
	II	$6{\cdot}6 \times 10^5$

φX and G4-infected cells were pulse-labelled for 15 s during RF DNA replication with [*methyl*-^{3}H]thymidine. The cells were divided into 2 halves and lysed according to different procedures (see Materials and Methods). After centrifugation of the cell lysate the total amount of ^{3}H radioactivity in the supernatant fraction was determined.

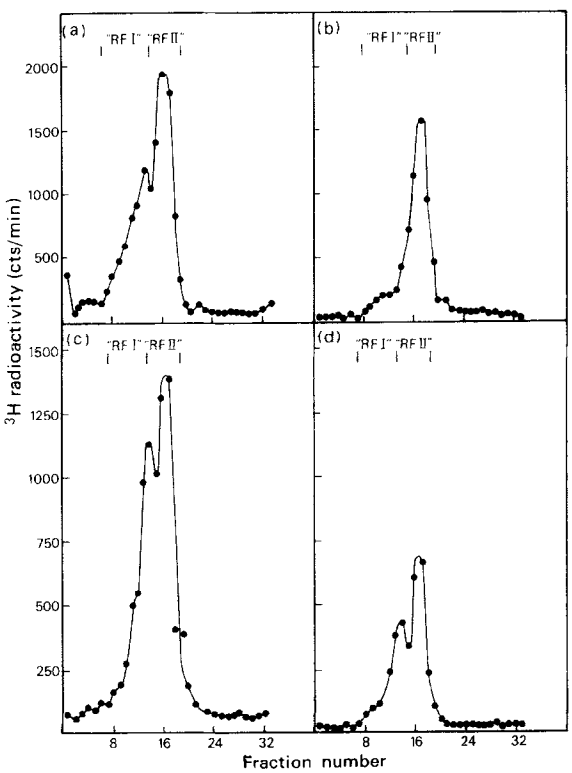

FIG. 2. Neutral high-salt sucrose gradient of φX and G4 DNA replicative intermediates, pulse-labelled for 15 s with [*methyl*-^{3}H]thymidine during RF DNA replication. The pulse was stopped by pouring the infected cells into an ethanol/phenol mixture. The cells were washed and DNA replicative intermediates were isolated using different procedures as described in Materials and Methods. After 19 h centrifugation at 24,000 revs/min at 15°C in a Beckman SW27 rotor, 10-μl samples of the fractionated gradient were counted.

(a) φX RF DNA replicative intermediates, isolated according to method I; (b) φX RF DNA replicative intermediates, isolated according to method II; (c) G4 RF DNA replicative intermediates, isolated according to method I; (d) G4 RF DNA replicative intermediates, isolated according to method II.

—●—●—, ^{3}H radioactivity (pulse-labelled φX or G4 DNA).

found for ϕX as well as for G4, when the cells were lysed according to method I. This indicates that using method II the great majority of the incorporated radioactivity was discarded with the pellet together with *E. coli* DNA. The difference of a factor of three between both methods was maintained during the further purification procedure. Sucrose density gradient centrifugation showed besides the quantitative also a qualitative difference (Fig. 2). Especially in the case of ϕX less material sediments

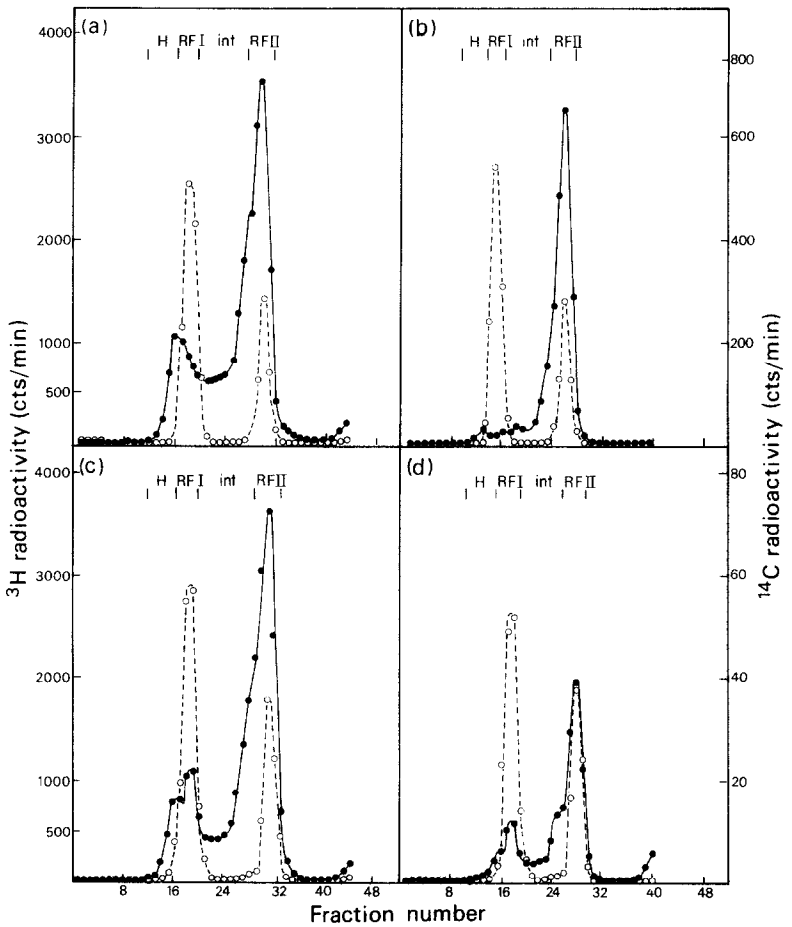

FIG. 3. Analytical PrI_2/CsCl gradients of ϕX and G4 DNA replicative intermediates, pulse-labelled for 15 s with [*methyl*-^{3}H]thymidine during RF DNA replication and isolated by different procedures. The "RFI" and "RFII" regions of the different sucrose gradients of Fig. 2 were collected as indicated. After dialysis against Tris/EDTA buffer and precipitation with isopropanol, equal samples of the "RFI" and "RFII" pool were mixed with ^{14}C-labelled ϕX RFI and RFII DNA ((a) and (b)) or with ^{14}C-labelled G4 RFI and RFII DNA ((c) and (d)).

(a) ϕX RF DNA replicative intermediates, isolated according to method I; (b) ϕX RF DNA replicative intermediates, isolated according to method II; (c) G4 RF DNA replicative intermediates, isolated according to method I; (d) G4 RF DNA replicative intermediates, isolated according to method II.

—●—●—, ^{3}H radioactivity (pulse-labelled ϕX or G4 DNA); --○--○--, ^{14}C radioactivity (uniformly labelled ϕX or G4 RF DNA).

H, heavy fraction; RFI, RFI fraction; Int, intermediate fraction; RFII, RFII fraction.

TABLE 2

Percentage of ^3H radioactivity of ϕX and G_4 RF DNA replicative intermediates found in the different fractions after PrI_2/CsCl equilibrium density centrifugation

Organism	Fraction	Isolation method Method I (%)	Method II (%)
ϕX	Heavy	13	3
	RFI	10	2
	Intermediate	29	23
	RFII	48	72
G4	Heavy	11	5
	RFI	12	17
	Intermediate	26	21
	RFII	51	57

ϕX and G4 RF DNA replicative intermediates, isolated by two different procedures, were separated in different fractions by PrI_2/CsCl equilibrium density centrifugation.

faster than RFII DNA when the cells were lysed according to method II. In this part of the gradient gapped molecules and rolling circles will sediment (Baas *et al.*, 1978). The "RFI" and "RFII" regions of the sucrose gradients were pooled separately, dialysed against Tris/EDTA buffer, concentrated by precipitation with isopropanol and subjected to preparative PrI_2/CsCl gradients (not shown). An equal sample of the RFI and RFII pool was mixed together with ^{14}C-labelled ϕX and G4 RFI and RFII DNA marker, respectively, and sedimented to equilibrium in an analytical PrI_2/CsCl gradient (Fig. 3). A quantitation of the radioactivity found in the various fractions

TABLE 3

Analytical BND-cellulose chromatography of ϕX and G_4 RF DNA replicative intermediates separated in different fractions by PrI_2/CsCl equilibrium density centrifugation

Organism	Fraction	Isolation method Method I (%)	Method II (%)
ϕX	Heavy	90	91
	RFI	68	52
	Intermediate	95	90
	RFII	64	53
	"RFII"†	82	76
G4	Heavy	87	89
	RFI	46	26
	Intermediate	89	89
	RFII	57	51
	"RFII"†	61	58

ϕX and G4 RF DNA replicative intermediates, isolated by two different procedures, were separated by PrI_2/CsCl equilibrium density centrifugation.

The figures represent the percentage of ^3H radioactivity found in single-stranded DNA containing replicative intermediates (caffein fraction).

† "RFII" is the RFII fraction, obtained from the preparative PrI_2/CsCl gradient from the "RFI" region of the sucrose gradient centrifugation. Approximately 1/3 of the total RFII fraction is present in "RFII".

of the PrI_2/CsCl gradients is shown in Table 2. For ϕX and G4 the same difference between the two methods was observed, although the difference was more pronounced in the case of ϕX. More DNA replicative intermediates were found in the heavy and intermediate fraction of the PrI_2/CsCl gradient, when isolation method I was used. This indicates that specifically DNA replicative intermediates with single-stranded regions were lost with isolation method II. This was confirmed by analytical BND-cellulose chromatography of the different fractions (Table 3). These results show that using method I many more DNA replicative intermediates have been isolated from

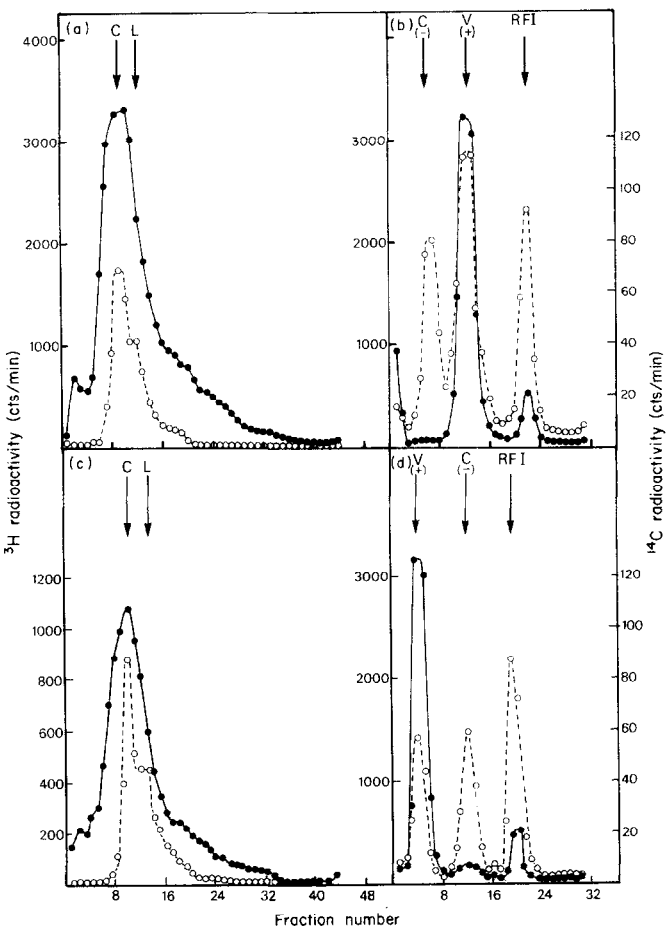

FIG. 4. Centrifugational analyses of the heavy fractions, isolated using method I after a 15-s pulse with [*methyl*-^{3}H]thymidine during RF DNA replication.

(a) Alkaline sucrose gradient centrifugation of the heavy fraction of ϕX; (b) poly(U,G)/CsCl gradient centrifugation of the heavy fraction of ϕX; (c) alkaline sucrose gradient centrifugation of the heavy fraction of G4; (d) poly(U,G)/CsCl gradient centrifugation of the heavy fraction of G4. —●—●—, ^{3}H radioactivity (pulse-labelled ϕX or G4 DNA). --○--○--, ^{14}C radioactivity (in the gradient of (a) ^{14}C-labelled circular (C) and linear (L) ϕX DNA were present; in the gradient of (b) ^{14}C-labelled ϕX RFI and RFII DNA; in the gradient of (c) ^{14}C-labelled circular (C) and linear (L) G4 DNA; and in the gradient of (d) ^{14}C-labelled G4 RFI and RFII DNA).

C (−), complementary strand DNA; V (+), viral strand DNA. Note, that the position of the complementary and viral strand DNA in poly(U,G)/CsCl gradients for ϕX and G4 is reversed.

the same quantity of infected cells than with method II. Therefore the further analyses were largely restricted to the DNA replicative intermediates obtained by method I.

(c) *Analysis of the heavy fractions*

In a previous paper (Baas *et al.*, 1978) we have shown that the heavy fraction of the PrI_2/CsCl gradient of ϕX DNA replicative intermediates consists of two types of molecules: rolling circles and RFI DNA with a low superhelix density. These two kinds of molecules can be separated by BND-cellulose chromatography. However, the amount of RFI DNA with low superhelix density is very low when the pulse-labelling is stopped by pouring the infected cells into an ethanol/phenol mixture (see also Table 3). Therefore the heavy fractions were analysed directly without further separation on BND-cellulose columns, by alkaline sucrose gradient and poly(U,G)/CsCl gradient centrifugation. Identical results were obtained for ϕX and G4. A total of 90% of the ^{3}H radioactivity in the heavy fractions was found as viral strand DNA longer than unit length (Fig. 4 and Table 4). This indicates that the great majority of the label in the heavy fractions is present in rolling circles.

(d) *Analysis of the RFI fractions*

The RFI fractions were passed through BND-cellulose columns to separate double-stranded RFI DNA from replicative intermediates containing single-stranded DNA regions. Poly(U,G)/CsCl and alkaline sucrose gradient centrifugation of the caffein fraction showed that a large part of this material consists of rolling circles (Fig. 5 and Table 4). The occurrence of a large percentage of small fragments may be explained by an overlap with the intermediate fraction (Figs 3 and 6) and some nicking

TABLE 4

Poly(U,G)/CsCl gradient analysis of the different fractions obtained after PrI_2/CsCl equilibrium density centrifugation

Organism	Fraction	Isolation method	
		Method I (%)	Method II (%)
ϕX	Heavy	93	96
	RFI double-stranded	23	N.D.
	RFI caffein	83	81
	Intermediate	70	53
	RFII	60	43
	"RFII"	66	64
G4	Heavy	92	91
	RFI double-stranded	14	N.D.
	RFI caffein	75	N.D.
	Intermediate	42	35
	RFII	28	29
	"RFII"	44	38

ϕX and G4 RF DNA replicative intermediates, isolated by two different procedures, were separated in different fractions by PrI_2/CsCl equilibrium density centrifugation. Based on the DNA sequence (Sanger *et al.*, 1978), uniformly [^{3}H]thymidine-labelled ϕX RF DNA contains 56·6% of the label in the viral strand and 43·4% in the complementary strand. For G4 the figures are 49·9% and 50·1%, respectively (Godson *et al.*, 1978).

The figures represent the percentage of ^{3}H radioactivity found in viral strand DNA.

N.D., not determined.

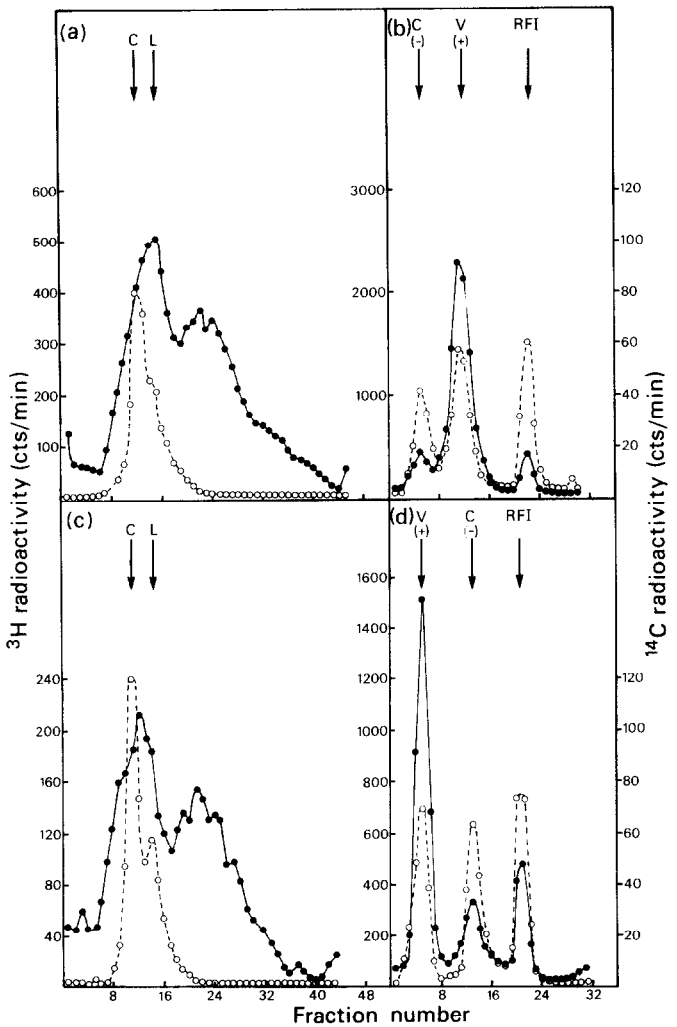

FIG. 5. Centrifugational analysis of the RFI DNA caffein fractions, isolated using method I after a 15-s pulse with [*methyl*-^{3}H]thymidine during RF DNA replication.

(a) Alkaline sucrose gradient centrifugation of the RFI DNA caffein fraction of ϕX; (b) poly-(U,G)/CsCl gradient centrifugation of the RFI DNA caffein fraction of ϕX; (c) alkaline sucrose gradient centrifugation of the RFI DNA caffein fraction of G4; (d) poly(U,G)/CsCl gradient centrifugation of the RFI DNA caffein fraction of G4.

—●—●—, ^{3}H radioactivity (pulse-labelled ϕX or G4 DNA). --○--○--, ^{14}C radioactivity (in the gradient of (a) ^{14}C-labelled circular (C) and linear (L) ϕX DNA were present; in the gradient of (b) ^{14}C-labelled ϕX RFI and RFII DNA; in the gradient of (c) ^{14}C-labelled circular (C) and linear (L) G4 DNA; and in the gradient of (d) ^{14}C-labelled G4 RFI and RFII DNA).

C (−), complementary strand DNA; V (+), viral strand DNA.

of the DNA molecules during BND-cellulose chromatography (routinely we have observed a conversion of approx. 50% from RFI into RFII DNA after BND-cellulose chromatography). Analysis of the double-stranded fractions obtained after BND-cellulose chromatography showed again identical results for ϕX and G4. The majority

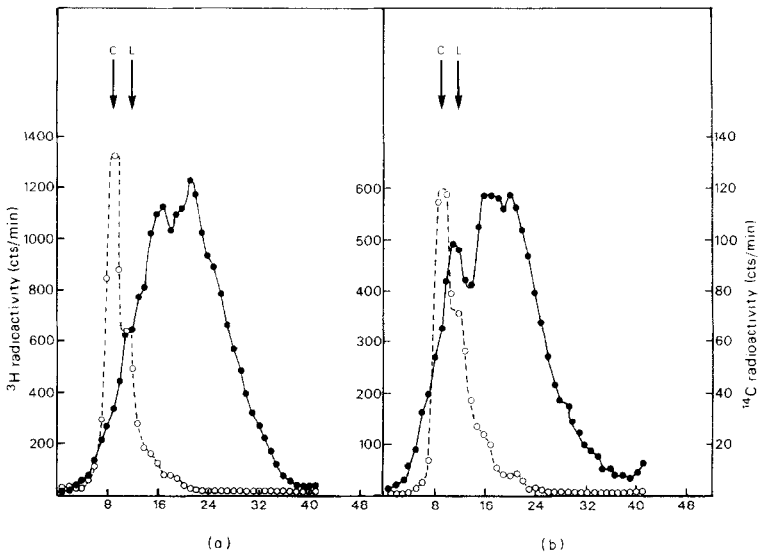

FIG. 6. Alkaline sucrose gradient centrifugation of the intermediate fractions, isolated using method I after a 15-s pulse with [*methyl*-^{3}H]thymidine during RF DNA replication.

(a) Alkaline sucrose gradient centrifugation of the intermediate fraction of ϕX; (b) alkaline sucrose gradient centrifugation of the intermediate fraction of G4.

—●—●—, ^{3}H radioactivity (pulse-labelled ϕX or G4 DNA). --○--○--, ^{14}C radioactivity (in the gradient of (a) ^{14}C-labelled circular (C) and linear (L) ϕX DNA were present; in the gradient of (b) ^{14}C-labelled circular (C) and linear (L) G4 DNA).

of the ^{3}H radioactivity in the RFI double-stranded fraction is found in complementary strand DNA (Table 4).

(e) *Analysis of the intermediate fractions*

Alkaline sucrose gradient analysis (Fig. 6) showed that the majority of the ^{3}H radioactivity in these fractions sediments as short fragments (8 to 11 S). More molecules sedimenting faster than unit length were observed in the case of G4. This presumably reflects an accidental fractionation of the PrI_2/CsCl gradient. Poly(U,G)/CsCl gradients showed that DNA replicative intermediates of the intermediate fraction, contain label both in the complementary and in the viral DNA strand (Table 4). The origin of short viral DNA fragments in relation to continuous or discontinuous synthesis of the viral DNA strand during RF DNA replication has been discussed previously (Baas *et al.*, 1978; Baas & Jansz, 1978; Hours *et al.*, 1978).

(f) *Analysis of the RFII fractions*

The isolation procedure followed resulted in two RFII fractions. One fraction was obtained from the preparative PrI_2/CsCl gradient of the RFII region of the sucrose gradient centrifugation (RFII fraction) and the other fraction was obtained from the preparative PrI_2/CsCl gradient of the RFI region of the sucrose gradient centrifugation ("RFII" fraction). Alkaline sucrose gradient centrifugation of the "RFII" fraction showed some molecules longer than unit length, some small fragments and a predominant peak at the position of linear ϕX and G4 DNA, respectively (not shown).

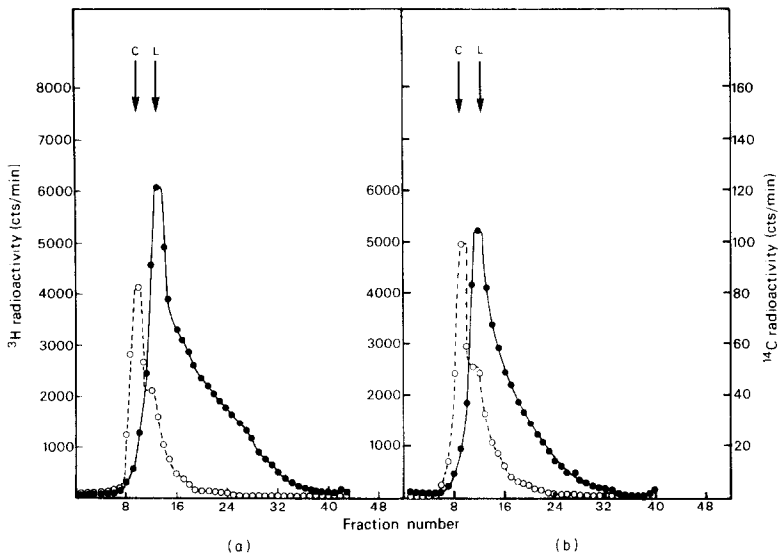

FIG. 7. Alkaline sucrose gradient centrifugation of the RFII fractions, isolated using method I after a 15-s pulse with [*methyl*-^{3}H]thymidine during RF DNA replication.

(a) Alkaline sucrose gradient centrifugation of the RFII fraction of ϕX; (b) alkaline sucrose gradient centrifugation of the RFII fraction of G4.

—●—●—, ^{3}H radioactivity (pulse-labelled ϕX or G4 DNA). --○--○--, ^{14}C radioactivity (in the gradient of (a) ^{14}C-labelled circular (C) and linear (L) ϕX DNA were present; in the gradient of (b) ^{14}C-labelled circular (C) and linear (L) G4 DNA).

The great majority of the RFII fraction sediments in alkaline sucrose gradients as linear ϕX and G4 DNA, respectively (Fig. 7). Poly(U,G)/CsCl gradient centrifugation of the RFII fractions showed that ^{3}H label was found in viral as well as in complementary strand DNA (Table 4).

(g) *Analysis of the DNA replicative intermediates obtained by isolation method II*

The qualitative analyses of the different fractions of the PrI_2/CsCl gradient following isolation method II, gave the same results as described above for the DNA replicative intermediates obtained by isolation method I, with one exception. The amount of ^{3}H label present in complementary strand DNA of DNA replicative intermediates was significantly higher than for the molecules isolated with method I (Table 4). In experiments with ϕX using shorter pulse times (5 to 10 s) we have observed that the amount of label found in complementary strand DNA of all DNA replicative intermediates can be as high as 70% (not shown). This indicates that using method II especially DNA replicative intermediates containing viral strand label, e.g. rolling circles and RFII DNA with a labelled viral strand and a non-radioactive complementary strand, were discarded with the pellet after centrifugation of the cell lysate.

(h) *Electron microscopic analysis*

Keegstra *et al.* (1979) have shown that the intermediate fraction of the PrI_2/CsCl gradient of ϕX DNA replicative intermediates contained besides single-stranded

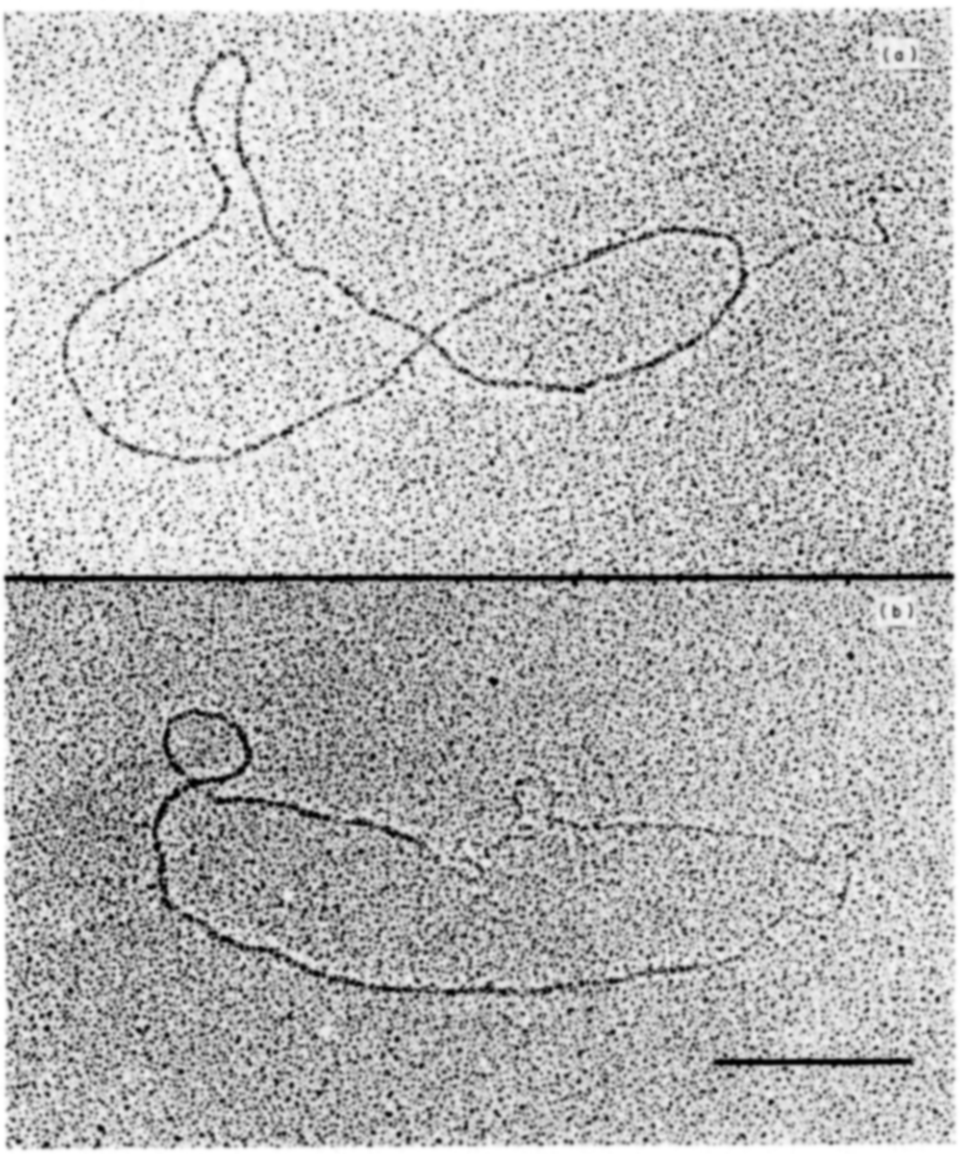

FIG. 8. G4 DNA rolling circle (a) and G4 partially duplex DNA molecule (b), isolated during RF DNA replication and observed in the intermediate fraction after PrI_2/CsCl gradient fractionation. The bar indicates 0·2 μm.

DNA, rolling circles and gapped molecules. The same types of molecules were observed in the electron microscope in the intermediate fraction of the G4 DNA replicative intermediates (Fig. 8). In the case of ϕX, one-quarter of the gapped molecules contained more than one single-stranded region (Keegstra *et al.*, 1979). In contrast,

the G4 gapped molecules contained only one single-stranded region, in agreement with the fact that G4 complementary strand DNA synthesis starts at a specific site (Martin & Godson, 1977; Hourcade & Dressler, 1978).

4. Discussion

Comparison of the biochemical analysis of ϕX and G4 DNA replicative intermediates, isolated by two different procedures during the period of RF DNA replications, have shown large quantitative and qualitative differences. However, no significant differences between the DNA replicative intermediates of ϕX and G4 were found when they were isolated using the same procedure. Lysis of the cells at a relatively high cell concentration (method II) resulted in a loss of a great part of the DNA replicative intermediates in the pellet after centrifugation of the cell lysate (Table 1). The possibility that this is caused by incomplete lysis of the cells using method II is not likely because of the qualitative and quantitative differences found in analysing the DNA replicative intermediates. The two isolation procedures differ only in the lysis method of the cells. After removal of *E. coli* DNA the purification and isolation of the DNA replicative intermediates takes place following the same procedure. Two observations indicate that especially DNA replicative intermediates with labelled viral strands, e.g. rolling circles and RFII DNA, containing a non-radioactive complementary strand circle and a linear radioactive viral strand, were lost. First, there is a reduction of the percentage of radioactivity in the DNA replicative intermediates found in the heavy fraction after PrI_2/CsCl gradient centrifugation (Fig. 3 and Table 2). Second, poly(U,G)/CsCl analysis of the different fractions shows an enrichment of DNA replicative intermediates with complementary strand DNA label, when isolation method II was used (Table 4). It has been mentioned earlier (Denhardt, 1977; Baas & Jansz, 1978) that DNA replicative intermediates may become associated with the membrane fraction, because of their single-stranded character or through their association with hydrophobic proteins. Apparently, the majority of the DNA replicative intermediates in method II is trapped in the membrane fraction during centrifugation of the cell lysate. It is not clear why DNA replicative intermediates, containing pulse-labelled viral strand DNA are specifically lost. Possible explanations are the amount and nature of the proteins in the replication complex (in method I a Pronase treatment is given before and in method II a Pronase treatment is given after centrifugation of the cell lysate) or the location of the DNA replicative intermediates within the cell. Viral strand DNA synthesis may take place at or in close vicinity of the cell membrane and the gap filling process of complementary strand DNA synthesis, after separation of the DNA replicative intermediates into two daughter molecules may take place elsewhere in the cell.

The biochemical analysis of G4 and ϕX DNA replicative intermediates, isolated by the same method, shows two minor differences. First, the amount of completed RFI DNA is larger for G4 than for ϕX. This may be connected with the shorter latent period of G4 and the specific initiation of G4 complementary strand DNA synthesis in contrast to ϕX. Second, in G4 RFII DNA much more label is present in complementary than in viral strand DNA (Table 4). This suggests also that using isolation method I, part of G4 RFII DNA with radioactive viral strands was lost after centrifugation of the cell lysate.

We have demonstrated by biochemical and electron microscopic analysis the presence of a significant amount of rolling circles amongst the DNA replicative intermediates during G4 RF DNA replication. Godson (1977*b*) has detected no rolling circles during G4 RF DNA replication. Our results suggest that he might have lost most, if not all, of the rolling circles during centrifugation of the cell lysate. However, G4 DNA rolling circles were observed during single-stranded DNA synthesis (stage III) using the same lysis and isolation procedure (Godson, 1977*a*). This may be explained by the enlargement of the number of rolling circles within the cell during stage III (Dressler & Denhardt, 1968; Koths & Dressler, 1978) and/or the localisation of the rolling circles within the cell during stage III. In this respect it may be relevant to note that Knippers & Sinsheimer (1968) have shown that pulse-labelled ϕX DNA during RF DNA replication, after gentle lysis of the cells, was found at the cell membrane and that during single-stranded DNA synthesis the radioactivity was present in the cytoplasm (Knippers *et al.*, 1969*b*).

ϕX and G4 code for the same series of proteins (Godson, 1974). Comparison of the amino acid sequence, derived from the DNA sequence (Sanger *et al.*, 1978; Godson *et al.*, 1978) shows a high degree of conservation between the ϕX and G4 proteins. In ϕX, the gene *A* protein is the only phage-coded protein required for RF DNA replication. Borrias *et al.* (1979) have reported that a functional G4 gene *A* protein is also needed for G4 RF DNA replication. They also have observed that an amber mutant in G4 gene *A* complements asymmetrically. Similar results have been obtained earlier for S13 (Tessman, 1965,1966) and ϕX gene *A* mutants (Lindqvist & Sinsheimer, 1967). Furthermore, *in vitro* studies have shown that the viral strand of supertwisted ϕX RFI and G4 RFI DNA is nicked by ϕX gene *A* protein at the same position, the origin of viral strand DNA replication (Langeveld *et al.*, 1978; van Mansfeld *et al.*, 1978). In both phages a stretch of 30 nucleotides around the position of the nick is completely conserved (Fiddes *et al.*, 1978; van Mansfeld *et al.*, 1978; Baas & Jansz, 1978). This suggests an identical function of the gene *A* proteins in the process of RF DNA replication of the two related phages, initiated by nicking of the viral strand at the origin of DNA replication. The biochemical and electron microscopic analysis of ϕX and G4 RF DNA replicative intermediates, described in this paper, also showed similar results for both phages. Therefore, we favour a replication model for G4 RF DNA replication, identical as proposed for ϕX RF DNA (Fig. 1), with one important difference. For ϕX, complementary strand DNA synthesis can start at many, although not at random sites (Eisenberg *et al.*, 1975; Keegstra *et al.*, 1979) but G4 uses a specific origin for the complementary strand DNA synthesis, which is located in the intergenic region between the genes *F* and *G* (Martin & Godson, 1977; Hourcade & Dressler, 1978).

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