Filamentous Bacterial Viruses

V. Asymmetric Replication of fd Duplex Deoxyribonucleic Acid

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Short pulses (30 sec at 32 C) of ^3H -thymidine were found primarily in the viral strands of replicating fd deoxyribonucleic acid (DNA), even at a time when most DNA being synthesized was duplex DNA. Much of the labeled viral strand DNA was longer than unit length, but some was shorter than unit length. Most of the corresponding complementary-strand DNA was recovered in closed supercoiled duplex molecules, even for short pulses; the remainder of the complementary-strand DNA was found in replicative intermediates in pieces shorter than unit length. Some of the viral strands in open replicating DNA lacked a corresponding complementary strand.

The fd-type bacterial viruses contain single-stranded circular deoxyribonucleic acid (DNA), weighing about 2×10^6 atomic mass units. Upon infection, a complementary strand is synthesized to form a circular duplex. This duplex replicates to give many progeny duplex molecules (24).

It is not clear how progeny single-stranded DNA is formed. One model would require a "single-strand-DNA polymerase" to make new single strands on a double-stranded DNA template. An alternative model (24) would require that the polymerization of single-stranded progeny DNA is carried out by the same enzymes that are used to polymerize double-strand DNA. The switch from double-strand to single-strand DNA synthesis would then require that polymerization of the complementary strand on one side of the DNA replication fork be inhibited, to displace new free single strands. The current evidence (29, 32) on the role of viral gene 5 in replication of single-stranded DNA is consistent with this second model, but does not prove it.

One prediction of this model is that replication of duplex DNA is in some sense asymmetric, since otherwise the mechanism which blocks synthesis of new complementary strands would also block synthesis of new viral strands. We report here studies on this asymmetric replication, using short pulses of ^3H -thymidine to label intermediates in replication.

MATERIALS AND METHODS

Media and solutions. N1 solution contained 0.5 g of NaCl, 8.0 g of KCl, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 12.1 g of tris(hydroxymethyl)aminomethane (Tris), 1 liter of water, 0.087 g of KH_2PO_4 , and 0.1 ml of 1 M CaCl_2 ; the pH was adjusted to 7.4 with HCl.

N11 medium was the same as N12 except that it contained only 0.023 g of KH_2PO_4 per liter.

N12 medium contained 1 liter of N1 solution and 1 ml of 1 M CaCl_2 , 5 ml of 0.2 g/ml NH_4Cl , 1 ml of 1 μg /ml FeCl_3 , 10 ml of 20% glucose, 10 ml of 5% Casamino Acids, and 5 ml of 2 mg/ml thiamine.

Pronase solution contained 10 mg of Pronase/ml in 0.2 M NaH_2PO_4 . A precipitate which formed after storage at 0 C was removed by low-speed centrifugation.

Tris-EDTA solution was 0.05 M Tris-0.003 M ethylenediaminetetraacetate, adjusted to pH 8.1 with HCl.

Chemicals. Mitomycin C was from Nutritional Biochemicals Corp.; Pronase, from Calbiochem; lysozyme, from Sigma Chemical Co.; sarkosyl NL97, from Geigy Chemicals; and thymidine-methyl- ^3H , thymine-methyl- ^3H , and ^{32}P -orthophosphoric acid (carrier-free), from New England Nuclear Corp.

Bacterial and virus strains. Bacterial strain DM133 is *uvrA*⁻, requires 2 μg of thymine/ml for growth, and lacks endonuclease I. It was formed by P1 transduction of the *endo I*⁻ locus from strain HfrC6 (8) into strain DM145, a male derivative of HF4704 (22), with *thy* used as the selection marker. The transductant was then made *Thy*⁻ by trimethoprim selection.

Wild-type fd was used for most experiments. Strain fd24 (*am5*) carries an amber mutation in gene 5 (24), and does not produce single-stranded progeny DNA in a nonsuppressing host (29).

Infection and DNA extraction. Strain DM133 was grown in medium N12 plus 5 μg of thymine/ml at 37 C to a density of 2×10^8 bacteria/ml. The bacteria were concentrated fivefold in N12 containing 2 μg of thymine/ml and 50 μg of mitomycin C/ml, incubated for 10 min at 37 C, washed twice with N1, and resuspended in N12 plus 2 μg of thymine/ml. The culture was incubated for 2 min at 32 C before addition of fd at a multiplicity of 500 plaque-forming units/bacterium. The infected culture was labeled for

a short time with ^3H -thymidine. Replication was stopped by pipetting a sample into a large flask immersed in dry ice-acetone mixture (6). The culture was then thawed in the presence of 0.01 M KCN and collected by centrifugation at 5,000 rev/min for 5 min at 4°C in a Sorvall SS-34 rotor. The bacteria were washed once with 0.8% NaCl-Tris-EDTA solution and resuspended in Tris-EDTA at a concentration no greater than 5×10^9 bacteria/ml.

The DNA was extracted by treating the bacteria with 0.01 M EDTA and 100 μg of lysozyme/ml for 15 min at 0°C followed by 1% sarkosyl and 500 μg of Pronase/ml from the stock solution. The extract was then incubated for 2 hr at 37°C.

Analysis by centrifugation. For velocity band centrifugation, samples were layered on 5 to 20% (w/v) sucrose gradients prepared with one of the following solutions. High-salt neutral velocity gradients were prepared with 0.5 M NaCl, 0.05 M Tris, and 0.003 M EDTA, pH 8. Low-salt neutral velocity gradients were prepared with 0.01 M Tris and 0.003 M Na₂EDTA, pH 8. Alkaline velocity gradients were prepared with 0.3 N KOH, 0.7 M KCl, and 0.003 M EDTA. Alkaline velocity gradients using the SW 40 rotor were underlayered with 0.7 ml of 20% alkaline sucrose, saturated with CsCl. Neutral equilibrium gradients contained 2.65 g of CsCl and 2.0 g of sample in Tris-EDTA. Alkaline equilibrium gradients contained 4.0 g of CsCl and 2.9 g of sample in 0.05 M Na₂PO₄-0.003 M EDTA, adjusted to pH 12.5 with 1 N NaOH. The alkaline equilibrium gradients were centrifuged in polyallomer tubes.

Gradients which were not to be analyzed further were collected on Whatman GF/A filters. Gradients which were to be analyzed further were collected in Analocups (Aloe Scientific), and portions were measured on GF/A filters. Samples from the low-salt neutral velocity gradients were precipitated with 20 μg of denatured salmon sperm DNA carrier per ml by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The precipitate was stored at -20°C overnight, collected by centrifugation at 12,000 rev/min for 30 min at 4°C in a Sorvall SS-34 centrifuge, and redissolved in 0.3 ml of 0.3 N KOH for recentrifugation on alkaline velocity gradients. Between 70 and 80% of the radioactivity was recovered from the ethanol precipitation. Samples from the alkaline velocity gradients were added directly to the alkaline CsCl solution for further analysis by alkaline equilibrium centrifugation.

Fractions were washed with 10% trichloroacetic acid and acetone and were counted by use of low-potassium-glass (Packard Instrument Co.) or nylon (Nuclear-Chicago Corp.) scintillation vials to lower the level of background radioactivity. In general, samples were counted for 10 min. When the amount of radioactivity was low (as in Fig. 2-5), samples were counted for at least 30 min.

The graphs of radioactivity against fraction number were divided into regions on the basis of the positions of maxima on the graphs. The total radioactivity in each region was determined by integrating the radioactivity in the region after subtracting a base-line level of radioactivity. When DNA from one region was

pooled and analyzed by a further centrifugation experiment, the radioactivity was normalized to the first gradient. When the viral and complementary strands were separated by alkaline equilibrium gradient centrifugation, the radioactivity of the complementary strand region was multiplied by 1.4 to correct for the fact (24) that the percentage of thymine nucleotides in the viral strand (34%) was greater than the percentage of thymine nucleotides in the complementary strand (24%).

Definition of markers. The position of pulse-labeled DNA in the gradients was defined with respect to the position of standard ^{32}P -markers of known sedimentation behavior. Form II, the relaxed circular duplex with at least one strand nicked, was used as a marker in high-salt velocity centrifugation, where it has a sedimentation coefficient of 19S (31). Viral single-stranded marker (V) has a sedimentation coefficient of 29S under these conditions. Form I, the supercoiled duplex with both strands covalently closed, has a sedimentation coefficient of 24S in high salt.

The sedimentation coefficients in low-salt velocity centrifugation, calculated relative to form II in a parallel high-salt gradient, are 13S for marker V, 25S for form I, and 18S for form II.

Linear marker V, obtained by one-hit ^{60}Co irradiation of circular V, was used as the sedimentation reference in alkaline velocity centrifugation, where it has a sedimentation coefficient of 18S (26).

The weight of the pulse-labeled DNA was calculated from the sedimentation coefficients by use of the Studier equations (38).

The viral single-stranded marker, V, was used as the reference in alkaline equilibrium gradients, where its density is 1.763 g/cm³ (30, 40). The term VS (viral strand) refers to labeled material which was found at the position of marker V in alkaline equilibrium centrifugation. CS (complementary strand) is labeled material found slightly above marker V, at 1.748 g/cm³ (30, 40), the density of the complementary strand. No assumption about the size of the molecules is implied by these terms. "Unit length" refers to material which has the same mass as viral single-stranded DNA, 2×10^6 atomic mass units. In general, we have assumed that material which has the same sedimentation coefficient as marker V in alkaline velocity centrifugation has the same mass as marker V. When marker V or C was degraded to half unit length, the buoyant density of the markers was unchanged. Therefore, the four bases are uniformly distributed throughout the molecule at this level of resolution.

Preparation of markers. Markers were prepared from virus or from infected bacteria grown in N11 containing 0.08 mCi of ^{32}P -orthophosphate/ml. The virions were passed through a column of Sephadex G-100 in 0.01 M Tris, pH 7.4, and were purified by equilibrium banding (39). Marker V was isolated from purified virions by phenol extraction (25). Form I was isolated from infected cells by removing bacterial DNA in a sodium dodecyl sulfate (SDS) precipitate (33) and recovering the viral DNA from a high-salt neutral velocity gradient. Single-stranded DNA was

FIG. 1. Low-salt neutral velocity centrifugation analysis of DNA after infection with fd. Mitomycin C-treated bacteria were infected with fd at 32 C. At 10 min after infection, 10 μCi of ^3H -thymidine/ml was added, and samples were taken after 30, 60, 90, and 120 sec. The extracted DNA was analyzed by low-salt neutral velocity centrifugation in a Spinco SW 27 rotor at 23,000 rev/min for 13 hr at 18 C. The samples included ^{32}P -fd form I, form II, and V marker (from left to right, indicated by arrows). The regions indicated by bars were pooled for further analysis. In the 30-sec sample, region α includes fractions 13–19 and region γ includes fractions 20–22. In the 60-sec sample, region α includes fractions 13–16, region β includes fractions 17–20, and region γ includes fractions 21–23. In the

also removed by the SDS precipitate. Form II was prepared by irradiating form I in a ^{60}Co source (11) to give an average of one to three hits per molecule. Similarly, a mixture of circular and linear unit-length V marker was prepared by ^{60}Co -irradiation of circular V to give two hits per molecule and was then isolated as unit-length DNA from a high-salt neutral velocity gradient. The markers were irradiated in Tris-EDTA, and were stored frozen in the same solution.

RESULTS

Outline of the pulse experiment. An asynchronously replicating population of viral DNA molecules was labeled with successively longer pulses of ^3H -thymidine, where the shortest pulse length was similar to the time needed to replicate one viral DNA molecule (a round of replication). The radioactive DNA was analyzed by neutral velocity band centrifugation to separate intermediates with different mass or frictional coefficients. The separated intermediates were each analyzed further by alkaline velocity band centrifugation, to separate the two strands of the DNA. Finally, the separated strands were analyzed by alkaline equilibrium centrifugation, to determine the relative amounts of radioactivity in the two halves of the duplex DNA.

To estimate the length of the round of replication, we used the fact that the growth point of the *Escherichia coli* chromosome replicates at 500 to 700 nucleotide pairs per sec at 37 C, and 150 to 210 nucleotide pairs per sec at 20 C (23). We estimated from these numbers that the fd genome replicates in about 10 sec at 37 C or 15 sec at 32 C. Measurement of ^3H -thymine incorporated into mitomycin C-treated bacteria infected with fd at 32 C confirmed this calculation: the rate of incorporation corresponded to a replication time of 30 sec per fd genome at 10 min after infection. This number may be high, because not all bacteria are infected.

Pulse labeling of fd duplexes. Radioactive thymidine was added to infected mitomycin C-treated bacteria at 10 min after infection at 32 C, and samples were withdrawn 30, 60, 90, and 120 sec later for analysis by low-salt neutral velocity centrifugation (Fig. 1). A large fraction of the

90-sec sample, region α includes fractions 13–17, region β includes fractions 18–21, and region γ includes fractions 22–24. In the 120-sec sample, region α includes fractions 12–16, region β includes fractions 17–19, and region γ includes fractions 20–22. Sample volumes of 30, 20, 10, and 10 ml were taken for the 30-, 60-, 90-, and 120-sec pulse lengths, respectively. Equal portions of each fraction were measured for radioactivity. However, for the 30- and 60-sec samples in this figure and in Fig. 2, only one-third and one-half (respectively) of the measured radioactivity was plotted, to account for the greater sample volume.

replicative intermediates which were labeled during a 30-sec pulse were found in region γ of the gradient, at the position of marker form II, and further intermediates were found between marker form I and marker form II. As the pulse length was increased, an increasing fraction of the radioactive DNA appeared in region α , at the position of marker form I. A similar pattern of labeling with increased pulse length (label first in the marker form II region, then increasingly in marker form I region) was found during times at which most DNA being synthesized is single strands (10, 30).

Analysis on alkali gradients. The low-salt gradients were subdivided into three regions as shown in Fig. 1 (the amount of radioactivity incorporated during a 30-sec pulse was small, so regions α and β were combined), and the pooled material from each region was analyzed further by alkaline velocity centrifugation, to determine the relative sedimentation coefficients of the individual strands of the labeled intermediates (Fig. 2). After a 60-sec pulse, region α (Fig. 2a) contained DNA (α_1) which had a sedimentation coefficient greater than that of circular marker, and also contained

DNA at the bottom of the gradient, presumably denatured supercoils (form I). At later times, the single-stranded DNA of region α was resolved into discrete peaks at the positions of linear and circular markers. Region β (Fig. 2b) was especially rich in DNA with the sedimentation coefficient of linear marker after a 60-sec pulse, but also contained DNA spreading towards higher sedimentation coefficients. We assumed that this faster-sedimenting DNA is larger than unit linear DNA molecules. With longer pulses, an increasing fraction of the DNA sedimented with circular marker, giving two resolved peaks. Region β , like region α , contained DNA which sedimented rapidly in alkali, as expected for supercoils. These experiments indicate that radioactive precursors are incorporated into the replicative intermediates of regions α and β first into linear strands, in part longer than unit length, and only later into circular strands.

The radioactivity in region γ (Fig. 2c) was mostly in linear DNA after short pulses, but increasingly in circular DNA after long pulses. No supercoils were apparent in this region.

Analysis of strand specificity in the 60-sec pulse.

FIG. 2. Alkaline velocity centrifugation analysis of regions α , β , and γ of Fig. 1. The fractions comprising each of the regions shown on Fig. 1 were pooled, precipitated with ethanol, and resuspended in 0.3 ml of 0.3 N KOH. To each of the samples, ^{32}P -fd circular and linear V marker was added. The samples were analyzed by alkaline velocity centrifugation in a Spinco SW 40 rotor at 40,000 rev/min for 5.5 hr at 18 $^{\circ}C$. Between 42 and 45 fractions were collected, of which fractions 1 to 35 are shown; the radioactivity in the remaining fractions was at the level of background. (a) Region α of the 30-, 60-, 90-, and 120-sec samples of Fig. 1 (b) Region β of the 60-, 90-, and 120-sec samples of Fig. 1. (c) Region γ of the 30-, 60-, 90-, and 120-sec samples of Fig. 1. 3H pulse, $\bullet$; ^{32}P marker, $\circ$.

The alkaline velocity gradients of the 60-sec pulse-labeled DNA of regions α and β were subdivided further into α_1 and α_2 , β_1 , β_2 , and β_3 , as shown in Fig. 2a and 2b. The pooled material from each region was analyzed by alkaline equilibrium centrifugation, to determine the relative amounts of CS and VS (Fig. 3 and Fig. 4). DNA from region α_1 , which has a sedimentation coefficient of 25S, greater than that of circular marker, had the buoyant density of V marker (Fig. 3a). DNA from region α_2 , which has a sedimentation coefficient of 18S, that of linear marker, was found in part at the buoyant density of V marker, and in part at the buoyant density of CS, lighter than V marker (Fig. 3b).

DNA from region β_1 has a sedimentation coefficient about 20S, corresponding to a weight about 1.5 times larger than unit-length linear DNA. It had the buoyant density of V marker (Fig. 4a), as found for DNA from region α_1 . DNA from region β_2 , which has the sedimentation coefficient of linear marker, 18S, also had in part the buoyant density of V marker and in part the buoyant density of CS (Fig. 4b). DNA from region β_3 , however, which has a sedimentation coefficient of about 15S, less than that of linear marker, contained considerable DNA at the buoyant density of CS, as well as DNA at the buoyant density of V marker (Fig. 4c).

This analysis indicates that the DNA from

FIG. 3. Alkaline equilibrium centrifugation analysis of regions α_1 and α_2 of the 60-sec sample of Fig. 2a. The fractions comprising regions α_1 (fractions 18–20) and α_2 (fractions 24 and 25) of the 60-sec sample of Fig. 2a were pooled and analyzed by alkaline equilibrium centrifugation in a Spinco no. 50 angle-head rotor at 40,000 rev/min for 60 hr at 18 C. The samples included ^{32}P -fd V marker. (a) Region α_1 from the 60-sec sample of Fig. 2a. (b) Region α_2 from the 60-sec sample of Fig. 2a. ^3H pulse, $\bullet$; ^{32}P marker, $\circ$.

FIG. 4. Alkaline equilibrium centrifugation analysis of regions β_1 , β_2 , and β_3 of the 60-sec sample of Fig. 2b. The fractions comprising regions β_1 (fractions 18–20), β_2 (fractions 23 and 24), and β_3 (fractions 26–28) of the 60-sec sample of Fig. 2b were pooled and analyzed by alkaline equilibrium centrifugation in a Spinco no. 50 angle-head rotor at 40,000 rev/min for 60 hr at 18 C. The samples included ^{32}P -fd V marker. (a) Region β_1 from the 60-sec sample of Fig. 2b. (b) Region β_2 from the 60-sec sample of Fig. 2b. (c) Region β_3 from the 60-sec sample of Fig. 2b. ^3H pulse, $\bullet$; ^{32}P marker, $\circ$.

regions α and β of the 60-sec samples, shown in Fig. 2, is rich in linear viral strands which have sedimentation coefficients between 12S and 25S, and are therefore in part longer than unit length. The labeled complementary strands have sedimentation coefficients between 12S and 18S. The values for the integrated radioactivity (Table 1) show that the separable strands of regions α and β comprise 20% of the total radioactivity incorporated during a 60-sec pulse, and, of this 20%, over 70% is VS.

The sedimentation coefficient of the DNA from the β region of Fig. 1 was increased, relative to form I marker, by centrifugation in the presence of high salt. This region was rich in long VS. The single-strand regions of the intermediates, which are presumably responsible for the sedimentation behavior, may be uncomplemented VS DNA (16).

Analysis of strand specificity in region γ . The circular and linear regions in the alkaline velocity gradient analysis of region γ (Fig. 2c) were

TABLE 1. Integrated radioactivity in various classes of DNA^a

Pulse length (sec)	Region	Radioactivity (counts/min)				
		Total	Form I ^b	Faster than unit linear ^c	Unit linear ^d	Slower than unit linear ^e
30	α	360	50	150	110	50
	γ	370	30	60	200	80
60				(30, 30; 40)	(145, 55; 80)	(65, 15; 20)
	α	580	440	90	50	
				(90, 0)	(35, 15; 20)	
	β	490	160	130	130	70
	γ	720	70	(130, 0)	(95, 35; 50)	(35, 35; 50)
90				130	450	70
				(55, 75; 105)	(370, 80; 110)	
	α	1,050	680	230	140	
	β	860	390	220	250	
	γ	1,460	70	450	940	
120				(170, 280; 390)	(725, 225; 315)	
	α	1,870	1,200	420	250	
	β	1,100	450	400	250	
	γ	2,000	160	640	1200	
				(390, 250; 350)	(625, 575; 805)	

^a The radioactivity in each of the regions of the Fig. 1 gradients (α , γ of 30 sec; α , β , γ of 60 sec; etc.) was integrated, after subtracting background, and tabulated in the column headed "Total." Samples from each of these regions were analyzed further by alkaline velocity centrifugation (Fig. 2). The radioactivity in each of the regions of the Fig. 2 gradients was then integrated, and the fraction of the radioactivity recovered from each region was multiplied by the value in the "total" column, to give the values in the other columns. When the material from Fig. 2 was analyzed further by alkaline equilibrium centrifugation (Fig. 3-5), the radioactivity in the viral and complementary regions was integrated, and the fraction in each region was determined. This fraction was multiplied by the value tabulated for normalized Fig. 2 counts. This product is shown in parentheses, as (V, C; C $\times$ 1.4). For example, in region γ of the 120-sec pulse, there were 2,000 counts/min recovered from the gradient of Fig. 1. When this region was analyzed further by alkaline velocity centrifugation, 60% of the radioactivity was found at the position of unit linear marker, so 1,200 is tabulated in that column. When this material was analyzed further by alkaline equilibrium centrifugation, 52% of the radioactivity was found at the position of viral marker, and 48% at complementary strand position, so (625, 575; 805) is tabulated under 1,200.

^b Form I is the material at the bottom of the gradients. Although the peak was not clearly resolved in these gradients, similar values were found in other experiments.

^c Faster than unit linear includes circular DNA; it is the DNA found slightly faster than circular marker.

^d Unit linear is the DNA found at the position of linear marker.

^e Slower than unit linear is the DNA found slightly slower than linear marker.

analyzed further by alkaline CsCl equilibrium centrifugation, to determine the relative amounts of VS and CS (Fig. 5). The results are summarized in Fig. 6. Most of the labeled DNA was VS, and most of this was linear over the first minute of labeling. Circular VS was the last species to appear. After 30 sec, region γ was especially rich in VS, but by 120 sec the amount of VS roughly equaled the amount of CS.

Analysis of replicating DNA in am5-infected bacteria. Single-stranded progeny DNA appears in the bacteria relatively early after infection, although initially in only small amounts (10). Therefore, any analysis of replicating duplex DNA may be complicated by the presence of replicating single-stranded DNA. This problem

may be avoided by using a virus strain which carries an *amber* mutation in gene 5, and therefore does not permit accumulation of progeny single strands.

An experiment of this type is illustrated in Fig. 7 and 8. Radioactive thymidine was added at 10 min after infection by *am5* at 37 C, and samples were removed after 20 and 60 sec for analysis by high-salt velocity gradient centrifugation. In the fast-sedimenting region (indicated by a bar in Fig. 7), a larger fraction of the DNA was labeled during the shorter pulse than during the longer pulse, suggesting that this region may be enriched in replicative intermediates. This region was isolated from the high-salt neutral gradient and investigated further (Fig. 8). Parallel high-salt

FIG. 5. Alkaline equilibrium centrifugation analysis of regions γ_1 and γ_2 of Fig. 2c. The fractions comprising regions γ_1 (fractions 20 and 21) and γ_2 (fractions 23 and 24) of the 30-sec sample, regions γ_1 (fractions 21 and 22) and γ_2 (fractions 24 and 25) of the 60-sec sample, regions γ_1 (fractions 19 and 20) and γ_2 (fractions 22 and 23) of the 90-sec sample, and regions γ_1 (fractions 17 and 18) and γ_2 (fractions 20 and 21) of the 120-sec sample of Fig. 2c were pooled and analyzed by alkaline equilibrium centrifugation in a Spinco no. 50 angle-head rotor at 40,000 rev/min for 48 hr at 18 C. The samples included ^{32}P -fd V marker. (a) Regions γ_1 (circular) and γ_2 (linear) of the 30-sec sample. (b) Regions γ_1 and γ_2 of the 60-sec sample. (c) Regions γ_1 and γ_2 of the 90-sec sample. (d) Regions γ_1 and γ_2 of the 120-sec sample. ^3H pulse, $\bullet$; ^{32}P marker, $\circ$.

FIG. 6. Kinetics of incorporation into the individual strands of form II. The amount of radioactivity in each form of region γ is plotted against length of pulse, for the data of Table 1. Linear V ($\square$) is "unit linear," viral strand; circular V ($\circ$) is "faster than unit linear," viral strand; linear C ($\blacksquare$) is unit linear complementary strand, corrected for low thymine by multiplying with 1.4; circular C ($\bullet$) is faster than unit linear complementary strand, corrected by 1.4.

(Fig. 8a) and low-salt (Fig. 8b) gradients showed that an appreciable fraction of the presumed replicative intermediates had a sedimentation coefficient in high salt of 26S, faster than form I, but had a sedimentation coefficient in low salt of about 20S, slightly faster than form II. A large change in sedimentation coefficient with ionic strength is typical of single-stranded DNA. In a parallel experiment, the sedimentation coefficient of a V marker was 29S in high salt and 13S in low salt. Therefore, this experiment suggests that the presumed replicative intermediate molecules contain a large fraction of single-stranded DNA.

This same point is illustrated by a different type of experiment (Fig. 8c). In a neutral CsCl equilibrium gradient, the replicative intermediates were found at a buoyant density of 1.704 g/cm³, between a form I marker at 1.701 g/cm³ and a V marker at 1.722 g/cm³. Assuming that the buoyant density is proportional to the mass fraction of single-stranded DNA in the replicative intermediate (4), 15% of the intermediate is single stranded.

The replicative intermediates were also analyzed by alkaline velocity gradient centrifugation (Fig. 8d), which separates the DNA into its component single strands. Pulse-labeled DNA was observed both faster and slower than a unit-length circular marker. The faster DNA was shown by alkaline equilibrium centrifugation to be largely VS.

The form I region from Fig. 7 (the region around fraction 20) was analyzed by alkaline velocity gradient centrifugation, to separate supercoils, which move rapidly in alkaline centrifugation, from open forms, which may be fortuitously present in this region. A larger fraction of labeled form I region was found to be in open forms after the shorter pulse than after the longer pulse.

Predominance of duplex synthesis at 10 min after infection. The *am5* strain not only fails to make single-stranded DNA in nonsuppressing bacteria, but also it damages the physiology of the host (15), so it is not an ideal control for wild type. To confirm that virtually no single-stranded DNA was being made by 10 min after infection with

FIG. 7. High-salt neutral velocity centrifugation analysis of DNA after infection with *fd am5*. Mitomycin C-treated bacteria were infected with *fd24* at 37°C. At 10 min after infection, 10 μ Ci of ³H-thymidine/ml was added, and samples were taken after 20 and 60 sec. The extracted DNA was analyzed by high-salt neutral velocity gradient centrifugation in a Spinco SW 27 rotor at 22,000 rev/min for 15 hr at 4°C. The region indicated by a bar, fractions 15–18 of the 20-sec sample, was collected for further analysis (see Fig. 8). The two gradients are drawn superimposed, with the 60-sec sample shifted left by one fraction. Solid line, 20-sec incorporation; dashed line, 60-sec incorporation.

FIG. 8. Characterization of the replicating intermediates from Fig. 7. The region indicated by a bar in Fig. 7, fractions 15–18 of the 20-sec sample, was pooled, dialyzed against Tris-EDTA, and concentrated against Sephadex G-100. The sample was divided into four parts, which were analyzed as follows. (a) High-salt neutral velocity gradient centrifugation, and (b) low-salt neutral velocity gradient centrifugation in a Spinco SW 50.1 rotor at 35,000 rev/min for 3.5 hr at 18 C. The samples included ^{32}P -fd form I and form II markers. (c) Neutral equilibrium gradient centrifugation in a Spinco SW 50.1 rotor at 27,000 rev/min for 48 hr at 18 C. The samples included ^{32}P -fd V marker (SS) and form I marker (DS). (d) Alkaline velocity gradient centrifugation in a Spinco SW 50.1 rotor at 45,000 rev/min for 3 hr at 18 C. The samples included ^{32}P -fd circular V marker. ^3H pulse, $\bullet$; ^{32}P marker, $\circ$.

wild type at 32 C, we performed the pulse-chase experiment shown in Fig. 9. At 10 min after infection, ^3H -thymidine was added, and 30 sec later the radioactive thymidine was diluted with a 1,000-fold excess of unlabeled thymidine. At successive times after unlabeled thymidine was added, samples were taken for analysis by neutral high-salt velocity centrifugation. The supernatant

fluid from the bacteria in each case was precipitated with 10% trichloroacetic acid to determine the amount of acid-insoluble radioactivity (presumably free virions). Less than 2% of the radioactive DNA was found as intracellular single strands or in free virions after a 10-min chase.

When an intermediate in replication goes on to finish its current round of replication, it will segregate two radioactive duplex daughters if it was involved in duplex synthesis, or one radioactive duplex and one unlabeled single-strand if it was involved in single-strand synthesis. In the second case, a radioactive single strand will be displaced after a further round of replication. Since the total amount of viral DNA more than doubles (24) during the period of the 10-min chase, the replicating intermediates should undergo at least two further rounds of replication during the 10-min chase. The fact that no radioactive single strands are displaced during this time indicates that the labeled replicating intermediates are not involved in single-strand synthesis. After a 40-min chase, about 30% of the radioactive DNA was found in the supernatant fluid and about 10% was found as intracellular single strands, as expected if the viral strands of all duplex molecules are eventually displaced to form single-stranded DNA.

Another line of evidence showed that total synthesis is symmetric at 10 min after infection at 32 C. Analysis by an alkaline CsCl equilibrium gradient of total viral DNA labeled with a 60-sec pulse showed that equivalent amounts of VS and CS are synthesized. A similar result was obtained from a DNA-DNA hybridization experiment. The pulse-labeled DNA hybridized to reference denatured duplex and fd DNA in the same ratio as did uniformly labeled ^{14}C -thymine form I.

Segregation of daughter duplexes. The analysis of region γ summarized in Fig. 6 indicates enrichment of VS in form II molecules. However, the experiments in Fig. 7–9 show that this excess VS is not destined for single strands. Where is the CS that corresponds to this VS? Viral DNA was labeled in an experiment parallel to that shown in Fig. 1, for 60 and 120 sec, and the form I molecules were purified by alkaline sucrose velocity gradient centrifugation. After ^{60}Co irradiation to permit strand separation, the DNA was analyzed by alkaline CsCl centrifugation (Fig. 10). After a 60-sec pulse, the corrected ratio of CS to VS was 2.5 to 1, but by 120 sec the two strands were equally labeled. Apparently, the replicative intermediates are resolved asymmetrically into duplex daughters. One of the daughters, containing newly made CS, was found in form I; the other, containing newly made VS, was found in form II. The form II molecules must

FIG. 9. Pulse-chase in bacteria infected with *fd*. Mitomycin C-treated bacteria were infected with *fd* at 32°C. At 10 min after infection, 10 μ Ci of 3 H-thymidine/ml was added. After 30 sec, 2 mg of unlabeled thymidine/ml was added ("chase"), and samples were taken at 0, 10, and 40 min after the beginning of the chase. The bacteria were centrifuged, and the bacterial DNA was extracted and analyzed by low-salt neutral velocity centrifugation in a Spinco SW 50.1 rotor at 37,000 rev/min for 3.5 hr at 18°C. The supernatant fluid from the bacterial centrifugation was precipitated with 10% trichloroacetic acid to determine "supernatant radioactivity." The positions of form I, form II, and V markers are shown by arrows. An uninfected control was run through a parallel analysis. (a) Uninfected

FIG. 10. Analysis of form I. Mitomycin C-treated bacteria were infected with *fd* at 32°C. At 10 min after infection, 10 μ Ci of 3 H-thymidine/ml was added. After 60 and 120 sec, samples were taken for extraction of DNA. The DNA was made to 0.3 N KOH and analyzed by alkaline velocity centrifugation in a Spinco SW 27 rotor at 24,000 rev/min for 5 hr at 18°C. Fractions were collected, and a portion was taken to measure radioactivity. The form I region comprised 20% of the total radioactivity after a 60-sec pulse, and 40% after 120 sec. The form I peaks were precipitated with ethanol, dissolved in 0.4 ml of Tris-EDTA, and irradiated in a 60 Co source to give an average of two hits per molecule. The DNA was analyzed by alkaline equilibrium centrifugation in a Spinco no. 50 angle-head rotor at 40,000 rev/min for 48 hr at 18°C. 3 H pulse, $\bullet$; 32 P marker-V, $\circ$; (a) 60-sec pulse; (b) 120-sec pulse.

eventually also be converted to form I, since for longer periods of labeling the ratio of form I to form II in the bacteria increased. Similar enrichment of form I for CS has been found for ϕ X174 (7).

DISCUSSION

Replicating circular DNA molecules contain three necessary discontinuities in the duplex (24): the origin of replication, or initiation region; the replication points, or growing forks; and the

bacteria, 30-sec pulse, no chase. Total radioactivity, 18,292 counts/min; supernatant radioactivity, 52 counts/min. (c) Infected bacteria, 30-sec pulse, 10-min chase. Total radioactivity, 55,704 counts/min; supernatant radioactivity, 293 counts/min. (d) Infected bacteria, 30-sec pulse, 40-min chase. Total radioactivity, 57,254 counts/min; supernatant radioactivity, 14,421 counts/min.

terminus. The origin and the terminus may be both located at the same region of the polynucleotide chain, but the molecular structure of this region is different when replication terminates than when replication initiates. A topological requirement, the swivel (3), may be included in one of these discontinuities, or may form a further discontinuity. Between these discontinuities is smooth duplex DNA, which may be ignored when discussing replication.

Study of fd DNA replication is technically advantageous, because the entire replicating molecule can be isolated intact with relative ease. However, the replication point may be comparable in size (14, 28) to the whole genome, so the three discontinuities may not be resolved from one another. In analyzing experimental observations, it is important to distinguish between those aspects which bear on replication and those which bear on segregation of the two daughter DNA molecules after termination.

In our experiments, short pulses of ^3H -thymidine were incorporated preferentially into DNA molecules which have sedimentation coefficients distinct from those of both supercoils (form I) and relaxed supercoils (form II), and therefore differ from these circular molecules either in mass or in frictional coefficient or both. Three forms of DNA molecules more complex than circular DNA have been seen in the electron microscope: the Cairns or θ form (1, 3, 34), the rolling circle or σ form (9, 19, 35), and the replicating supercoil (17, 18, 36). These forms are related to one another by simple thought-experiments. The replicating supercoil can be converted to a Cairns form by a single-strand break in the supercoiled arm, and the Cairns form can be converted to the rolling circle by a break at one of the branch points (17, 21). In other words, these forms may differ only in the structure of the origin or terminus, and not in the structure of the replication point. The labeled material in our experiments which was not identical to either form I or form II probably has one of these more complicated structures, but we have not attempted to distinguish between the possibilities.

Asymmetric replication. DNA which is labeled with short pulses will be enriched in replicating intermediates. Analysis of such DNA, which sediments in regions α and β as open forms, shows that it is three times richer in VS than CS (Table 1). Much of the radioactive VS DNA, when separated from the intermediates in alkali, has a sedimentation coefficient greater than that of circular marker, suggesting that newly synthesized VS DNA is covalently attached to a pre-existing VS DNA (12, 41); however, some of the VS DNA has a sedimentation coefficient smaller

than that of linear marker (the range is 12S to 25S). On the other hand, all of the CS has sedimentation coefficients less than that of linear marker, ranging from 12S to 18S.

We have considered three possible explanations for the imbalance between VS and CS in regions α and β :

(i) The replicating DNA includes intermediates in single-strand replication (10, 30). This explanation is eliminated by the experiment shown in Fig. 9. No more than 2% of the 30-sec pulse label can be destined for single strands, but there is a 20% excess of VS over CS in regions α and β after a 60-sec pulse.

(ii) The intermediates may have already entered a second round of duplex replication, after having shed the first daughter which was labeled in the CS. On average, such structures would contain 1.5 units of VS and 0.5 unit of CS. In fact, region α contains VS which is 1.5 times unit length, but the amount of CS in this region is only one-sixth, not one-third, the amount of VS (Table 1).

(iii) Replicative intermediates contain uncomplemented VS. This hypothesis not only explains the asymmetry of VS and CS label, but also explains the sedimentation behavior of the material from region β as the ionic strength is varied (Fig. 8). From the data of Fig. 8, we estimate that there are between 1,000 and 5,000 uncomplemented nucleotides in the single-strand region of replicative intermediates. This is somewhat greater than the 100 to 1,300 nucleotides estimated for the single-strand regions at the replication point of λ bacteriophage (16). Single-strand regions have also been inferred for the replication points of bacteria (27, 28) and other viruses (5, 17).

Such single-strand regions may be a consequence of the asymmetric function of DNA polymerases, which advance only 3' ends of polynucleotide chains. The growing 5' end at the replication fork is thought to be advanced by short bursts of 3'→5' replication, as single strand regions are bared ahead of the advancing 5' end (28). The primer for these 3'→5' bursts could be an oligonucleotide (2, 28); it could be the advancing 3' ends on the other strand which periodically bridge the fork and replicate away from the fork (13, 20); or it could be prefork replication (14) of only the complementary strand. The last possibility could explain the longer than unit length complementary strands observed under some circumstances (19).

Asymmetric segregation of completed duplexes. After pulses of 60 sec or less, form I was found to be relatively rich in CS, and form II was rich in linear VS. This result suggests that the two

daughter duplexes arising from a replicating intermediate are not identical. The one containing new CS can be closed rapidly to form I, whereas the other remains as form II.

Presumably the replication machinery requires a specific form as its substrate: one or the other of the two asymmetric daughters. Assume that the substrate for further replication is the daughter which is recovered as form II. Then the unlabeled CS of form II would continually recycle through the replication machinery (Fig. 11). That is, replication would take place by a stamping machine mechanism (37) with the CS as master. A small number of these molecules (one per stamping machine) would be asymmetrically labeled even after very long pulses. The form I molecules, on the other hand, would become labeled in both strands after pulses longer than one replication cycle.

In fact, for pulses longer than 60 sec, form II molecules labeled in both strands were found (Fig. 6). The lag between the appearance of labeled linear VS and the appearance of labeled CS in form II would be expected if labeled CS appears because of random nicking of previously completed form I. The still greater lag before appearance of circular V could be expected if this DNA came from form I nicked in CS after a second round of replication (dotted line in Fig. 11). The fact that labeled circular strands appear in open structure of regions α and β after longer pulses (Fig. 2a and 2b) is consistent with the notion that nicked form I can feed back into the replication machinery.

FIG. 11. Asymmetric segregation of the two daughter duplexes after replication. Only one of the daughters can be used directly as a substrate by the replicating machinery. Therefore, one strand of this daughter has the characteristics of a stamping machine template, which is continually recycled through the replicating machinery. However, the other daughter might be converted (dashed arrow) to a form suitable for the replicating machinery.

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