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Replication of duplex DNA of phage φX174 reconstituted with purified enzymes

[viral (+) strand synthesis/complementary (−) strand synthesis/gene *A* protein/*rep* protein/prepriming proteins]

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ABSTRACT **Replication of the covalently closed duplex replicative form (RF) of phage φX174 DNA has been achieved by coupling two known enzyme systems: (*i*) synthesis of viral strand circles (SS) from RF, and (*ii*) conversion of SS to nearly complete RF (RF II). In this coupled system, activated RF (gene *A*·RF II complex) was a more efficient template and generated as many as 10 RF II molecules per RF input, at a rate commensurate with SS synthesis. The 11 proteins required for the two component systems were all needed in the coupled RF duplication system; no new factors were required. Single-stranded DNA binding protein was needed for RF duplication at only 4% the level needed in its stoichiometric participation in SS synthesis. In addition to RF II, more complex replicative forms appeared late in the reaction, and their possible origin is discussed.**

Replication of bacteriophage φX174 (φX) DNA in *Escherichia coli* proceeds by three stages: SS → RF, conversion of the single-stranded viral (+) circle (SS) to duplex replicative form (RF); RF → RF, multiplication of RF; and RF → SS, selective synthesis of viral strands for virions, using the complementary (−) strand of RF as template (1, 2).

Synthesis of the complementary strand *in vitro* to form RF II in the SS → RF stage depends on the host multiprotein DNA replicative system, which is probably identical to that utilized for discontinuous synthesis of the lagging strand in replication of the host chromosome (3–5). In this reaction, φX SS DNA is converted to a nucleoprotein intermediate that includes a tightly bound *dnaB* protein molecule and the single-stranded-DNA binding protein (SSB) (refs. 6–9; unpublished results). Formation of this prepriming intermediate requires participation of at least five additional *E. coli* proteins: proteins n, n′, n″, i, and the *dnaC* protein. The prepriming intermediate enables primase to synthesize a short primer that is extended by DNA polymerase III holoenzyme, leading to the formation of RF II, which is then converted to superhelical RF I by the successive actions of DNA polymerase I, DNA ligase, and DNA gyrase (unpublished observations).

Crude cell-free preparations for replication of RF I (RF → RF stage) (10, 11) have been fractionated into two separate systems (12, 13). One system (RF → SS) requires the participation of φX-encoded gene *A* protein, host *rep* protein, SSB, and DNA polymerase III holoenzyme to produce multiple copies of covalently closed, single-stranded viral circles from RF I. Replication is initiated by gene *A* protein, which creates a nick in the viral strand at the origin of RF replication (14–16). Synthesis by holoenzyme of viral strands, facilitated by helicase action of *rep* protein (17), proceeds by a looped rolling-circle mechanism (13).

The second system needed for RF synthesis is the initial reaction of complementary strand synthesis (SS → RF); the nascent viral strands now provide the template for complementary strand synthesis. Thus, viral and complementary strands are synthesized by distinct mechanisms (5, 14), analogous to the continuous and discontinuous mechanisms proposed in host chromosome synthesis (7, 14).

Although synthetic viral strands isolated from the RF → SS reaction were shown to serve as templates for the conversion of SS to RF (18), the reagents for the latter reaction strongly inhibited the RF → SS reaction. With more extensive purification of the numerous proteins required for both of these stages, coupling of the SS → RF with the RF → SS reactions has been achieved and is the subject of this report. Operation of the coupled system now provides an opportunity to investigate basic questions about the mechanisms of viral and complementary strand synthesis in RF duplication.

MATERIALS AND METHODS

Materials

Buffer A is 0.1 M Tris·HCl (pH 7.5), 20% (wt/vol) sucrose, 40 mM dithiothreitol, and bovine serum albumin at 0.2 mg/ml; buffer B is 0.1 M Tris·HCl (pH 8.1), 50 mM $MgCl_2$, 5 mM dithiothreitol, bovine serum albumin at 0.5 mg/ml, and 50% (vol/vol) glycerol; buffer C is 0.02 M Tris·HCl (pH 8.1), 1 M NaCl, 5 mM EDTA, bovine serum albumin at 0.1 mg/ml, 1 mM dithiothreitol, and 10% (vol/vol) glycerol.

Extensively purified *E. coli* replication proteins were: *rep* protein (4×10^7 units/mg) (19), gene *A* protein* (5.5×10^6 units/mg) (20), and dUTPase (2×10^7 units/mg) (18). The other replication proteins were as described (21). RNA polymerase holoenzyme (22) was kindly supplied by M. Chamberlin (University of California, Berkeley, CA) and DNA gyrase (23) by N. R. Cozzarelli (University of Chicago). φX DNA and RF I were prepared as described (24). *E. coli* tRNA was obtained from Boehringer Mannheim.

Methods

Assays of DNA Replication. *SS → RF reaction.* Components were added in the following order at 0°C: 5 μl of buffer A, 1.2 nmol each of dCTP, dGTP, dATP, and [α-^{32}P]dTTP (200 dpm/pmol), 2.5 nmol each of GTP, CTP, and UTP, 20 nmol of ATP, 0.2 μmol of $MgCl_2$, 250 pmol (as nucleotide) of φX DNA, 0.1 μg of *E. coli* tRNA, 10 ng of dUTPase, 1.5 μg of SSB, 0.4 μg of DNA polymerase III holoenzyme, 0.2 μg of *dnaB*

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Abbreviations: φX, phage φX174; SSB, single-stranded-DNA binding protein; SS, single-stranded circle of viral DNA; RF, duplex replicative form of DNA; RF I, double-stranded, covalently closed circular, superhelical DNA; RF II, double-stranded circular DNA with one or more single-strand breaks.

* Gene *A* protein used in this experiment was further purified by chromatography on blue dextran Sepharose 4B.

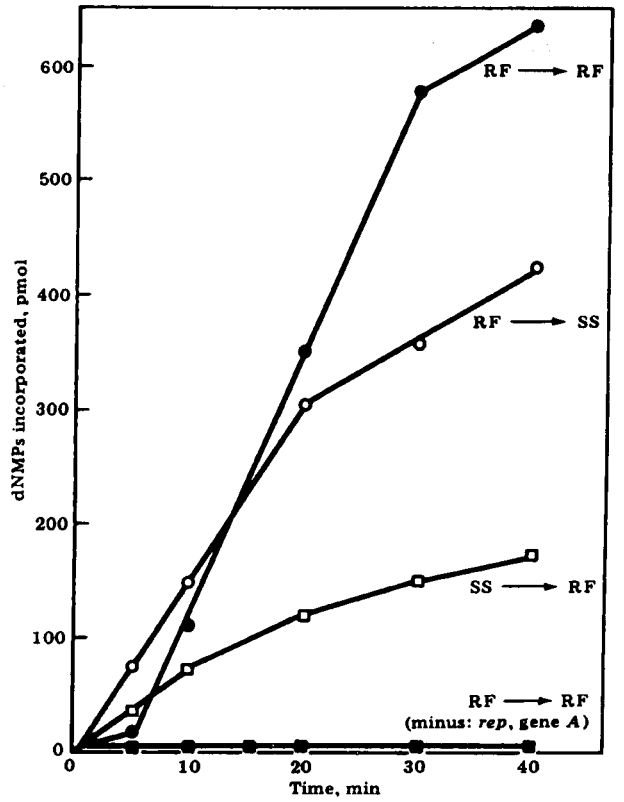

FIG. 1. Rate of DNA synthesis dependent on ϕX SS DNA or RF I as templates; 250 pmol of ϕX DNA or 180 pmol of RF I (as nucleotide) were used as templates. RF → RF reaction mixtures lacking gene *A* protein and *rep* protein served as controls.

protein, 50 ng of *dnaC* protein, 50 ng of protein i, 70 ng of proteins n + n″, 75 ng of protein n′, 80 ng of primase, and water to 25 μl.†

RF → SS reaction. Components were added in the following order at 0°C: 5 μl of buffer A, 1.2 nmol of dCTP, dGTP, dATP, and [α-^{32}P]dTTP (200 dpm/pmol), 2.5 nmol each of GTP, CTP, and UTP, 20 nmol of ATP, 0.2 μmol of $MgCl_2$, 180 pmol (as nucleotide) of ϕX RF I, 0.1 μg of *E. coli* tRNA, 10 ng of dUTPase, 10 ng of gene A protein, 25 ng of *rep* protein, 1.5 μg of SSB, and 0.4 μg of DNA polymerase III holoenzyme.†

RF → RF reaction. Components were added at 0°C as described for the RF → SS reaction. Then, proteins *dnaB*, *dnaC*, i, n + n″, n′, and primase were added in this order, as in the SS → RF reaction. Final volume was adjusted to 25 μl with water. When gene *A* protein·RF II complex was used as template, gene *A* protein and RF I were omitted from the reaction mixture. Incubations were at 30°C. At specified times, aliquots were analyzed for acid-insoluble radioactivity as described (25).

Gene *A* Protein·RF II Complex. Components were mixed at 0°C in the following order: 4 μl of buffer B, 5.5 μg of ϕX RF I, 0.9 μg of gene *A* protein, and water to 20 μl. Incubation was at 30°C for 20 min. Gene *A* protein·RF II complex was separated from unreacted gene *A* protein by chromatography on a Bio-Gel A-5m column (0.3 ml) equilibrated with buffer C at 4°C. Approximately 70% of the input RF I was converted to gene *A* protein·RF II complex under these conditions.

Agarose Gel Electrophoresis. For analysis of DNA products, the reaction was terminated by addition of 1% sodium dodecyl sulfate and samples were kept for 3 hr at 0°C. Electrophoresis was performed as described (26) on a gel bed, 16 × 17.7 cm, at an agarose concentration of 0.7% or 0.8%, in 0.089 M Tris/borate (pH 8.5) and 2.5 mM EDTA at room temperature for 4 hr, at a voltage of 1 or 2.5 V/cm, with a marker of bromophenol blue. DNA bands were stained with ethidium bromide; ^{32}P-labeled DNA was visualized by autoradiography.

RESULTS

Component Systems for Multiplication of RF. Conversion of RFI to single-stranded circles (RF → SS) yielded about five circles per input RF I (Fig. 1). This catalytic use of RF I required gene *A* protein, *rep* protein, and SSB (see below), as well as DNA polymerase III holoenzyme (12). The circles, identified autoradiographically after separation by agarose gel electrophoresis (Fig. 2), were the exclusive product of the reaction. The other system (SS → RF), conversion of single-stranded viral circles to RF, was nearly stoichiometric (Fig. 1) and depended on the multiple prepriming proteins, primase, and holoenzyme (21). As indicated by autoradiography and ethidium fluorescence after electrophoretic separation (data not shown), the product was RF II and was readily converted to supercoiled RF I by the successive actions of DNA polymerase I, *E. coli* DNA ligase, and *E. coli* DNA gyrase (unpublished observations). The RF → SS system was inert on SS, as was the SS → RF system on RF.

† The protein n fraction used here was further resolved into two components, protein n and n″. With highly purified components in this reaction mixture, tRNA and dUTPase were dispensable.

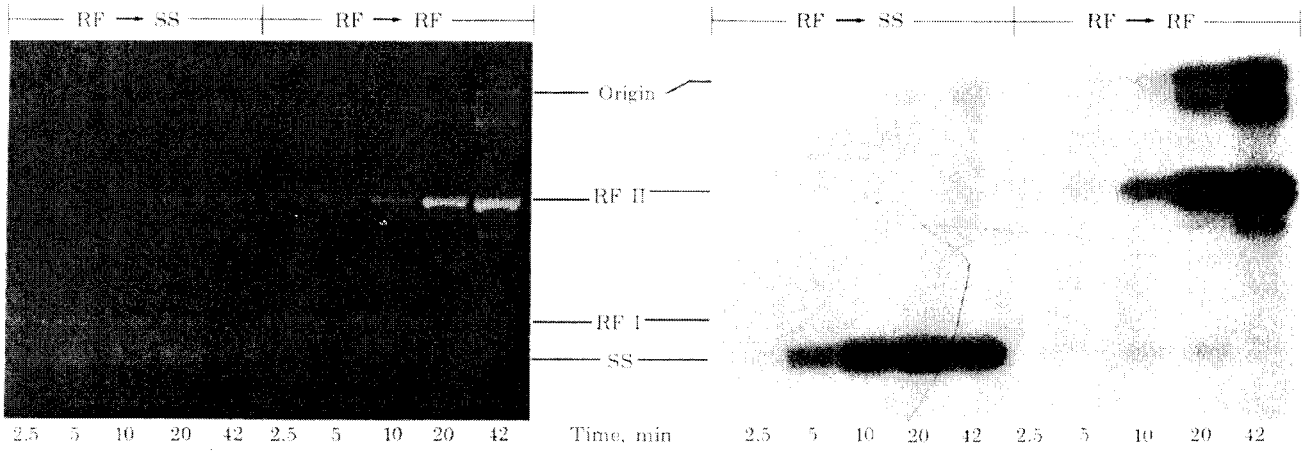

FIG. 2. Separation of DNA products by agarose gel electrophoresis. DNA syntheses (RF → RF, RF → SS) were carried out for the indicated times as described in *Materials and Methods* and in Fig. 1 legend. Aliquots (15 μl) treated with sodium dodecyl sulfate were subjected to agarose gel electrophoresis. The products were located by ethidium bromide staining (*Left*) and by autoradiography (*Right*).

Table 1. Requirements for DNA synthesis with purified proteins

Component omitted	dNMP incorporated, pmol
RF → SS	
None (complete)	420
SSB	10
Gene *A* protein	1
rep protein	1
RF → RF	
None (complete)	925
dnaB protein	288
dnaC protein	294
Proteins n + n″	152
Protein n′	388
Protein i	169
Primase	120
SSB	73
rep protein	2
Gene *A* protein	2
DNA polymerase III holoenzyme	1

DNA synthesis was measured with ϕX RF I as template. Incubation was 40 min.

When RF I and the components of both systems were combined, an augmented rate of DNA synthesis was observed after a brief lag (Fig. 1). The amount of product corresponded to about four RF molecules per input RF, indicating a catalytic use of the RF template. The principal product was RF II with virtually no single-stranded circles as shown by autoradiography and ethidium bromide fluorescence after agarose gel electrophoresis (Fig. 2). Thus, effective coupling of the RF → SS system to the SS → RF system was achieved under these conditions.

A complex form, which migrated slower than RF II or even failed to enter the agarose gel, was observed in the coupled reaction (Fig. 2). The nature of this slow-migrating, more complex form will be considered in the *Discussion*.

Prepriming and Priming Proteins of the SS → RF System are Required for RF Multiplication. Omission of any one of the proteins required for priming the SS → RF reaction reduced DNA synthesis in RF multiplication to a level of 13–35% of the coupled system (Table 1), and only single-stranded circles were formed (Fig. 3). (In the absence of gene *A* protein, *rep* protein, or SSB, DNA synthesis was nearly abolished.) The requirement for the prepriming and priming proteins of the ϕX system was not replaced by RNA polymerase (data not shown) despite the capacity of RNA polymerase to prime replication of single-stranded DNAs (27, 28).

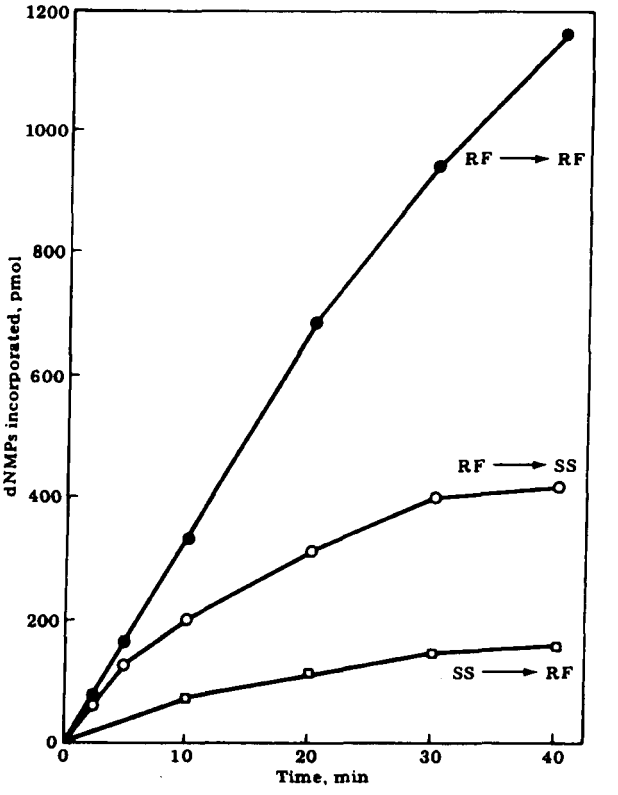

FIG. 4. Rate of DNA synthesis with gene *A* protein-RF II complex as template. DNA synthesis (RF → RF or RF → SS) was carried out as in *Materials and Methods* except that only 100 pmol (as nucleotide) of gene *A* protein-RF II complex was used. Stage I synthesis (SS → RF) was carried out with 250 pmol of ϕX DNA.

Gene *A* Protein·RF II Complex is a Superior Template in RF Replication. Because the nicking of RF I by gene *A* protein to form a covalent complex appeared to be the rate-limiting step in the RF → SS reaction (13), an isolated gene *A* protein-RF II complex proved to be a superior template in RF multiplication. The reaction started without a detectable lag, continued at a

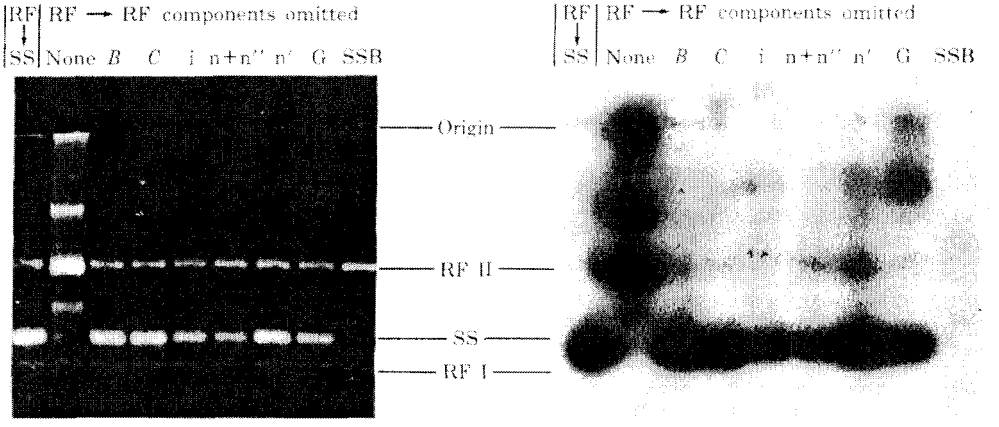

FIG. 3. Proteins required in the RF → RF reaction. DNA synthesis (RF → RF or RF → SS) was carried out as in *Materials and Methods* and in Table 1. Incubation was for 40 min at 30°C. DNA products were separated by agarose gel electrophoresis and located by staining with ethidium bromide (*Left*) or autoradiography (*Right*). The DNA products were analyzed in slots as indicated: *B*, *dnaB* protein; *C*, *dnaC* protein; G, primase.

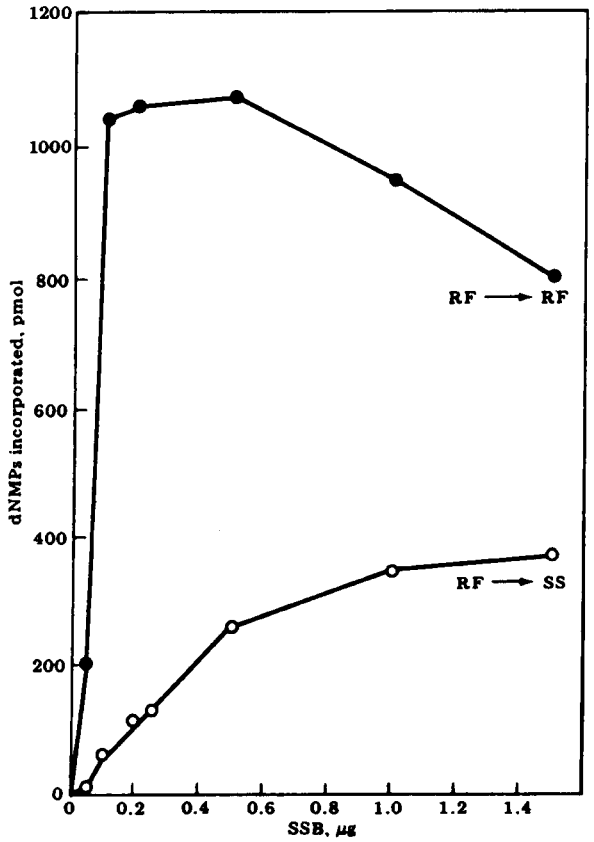

FIG. 5. Amounts of SSB required in RF → RF and RF → SS reactions. DNA synthesis was carried out with 180 pmol of RF I and varied amounts of SSB. Incubation was at 30°C for 40 min.

constant rate for more than 20 min, and after 40 min produced as many as 10 RF II molecules per starting complex (77% of the nucleotides were incorporated into RF II, 20.5% into the slow-moving component, and 2.5% into SS DNA) (Fig. 4). The products were similar to those observed in Fig. 2 when RF I was the template (data not shown).

Requirements for SSB in RF Multiplication. In the conversion of RF to SS, the stoichiometric requirements for SSB determines the extent rather than the rate of the reaction (17). Approximately 1 μg (13 pmol of the tetramer) of SSB was needed to produce 350 pmol of SS DNA (as nucleotide residues) (Fig. 5), an amount sufficient to cover the SS DNA, and near the reported values of 1 SSB per 40 nucleotides in SS DNA (25). In striking contrast, in the RF → RF reaction, only 0.1 μg of SSB was sufficient for the formation of over 1000 pmol of RF (Fig. 5); this amount of SSB (about 1 pmol) can cover only about half of the viral strand displaced from the starting RF I template (near 180 pmol). Whether the displaced viral strand is used as a template for complementary strand synthesis before the circle is completed or whether only half of the RF I molecules are active as templates is not yet clear.

DISCUSSION

Replication of the duplex circular form of φX DNA (RF) had been observed with crude enzyme fractions (10, 11). However, net quantities of RF had not been synthesized with such grossly impure preparations, nor could much be learned about the molecular details of the reaction. The facile synthesis of viral strand circles (SS) from RF with a gene *A* protein–*rep* protein system (12), and the capacity of the synthetic circles isolated from this reaction to serve as templates for RF synthesis by purified proteins suggested that coupling of the RF → SS system with the SS → RF system could account for multiplication of RF *in vivo*.

With an RF template and all the purified proteins needed to sustain the RF → SS and SS → RF reactions, a multifold replication of the RF template is observed (Figs. 1, 2, and 4). Earlier failures are attributable to inhibitory factors in less purified protein preparations. The need for dUTPase and tRNA observed in the partially purified system is not manifest when highly purified proteins are available. The coupled reactions proceed efficiently with the catalytic uses of RF template and SSB. The RF II product is readily converted to the supercoiled form upon addition of DNA polymerase I, ligase, and gyrase (unpublished observations). Thus it seems likely that, starting with the viral strand from virions, large quantities of RF can be formed by the combined actions of the purified SS → RF and RF → SS systems.

In addition to RF II, more complex forms, which included φX-unit-length duplex circles with multigenome-length tails accumulated late in the reaction. The long tails, which were double-stranded in some molecules and partially single-stranded in others, are thought to be byproducts of the reaction (L. Polder, personal communication). A plausible intepretation is that the tails arise from looped rolling-circle replicative intermediates in which the nicking-ligation step to produce a viral circle failed to take place. As a result the long displaced viral strand could become the template for SS → RF initiations that produce duplex regions. Further details about these molecules will be published elsewhere.

In the standard SS → RF reaction, multiple though nonrandom starts are made (7, 9). Protein n′ is presumed to have a special function in recognizing a unique sequence with a potential for hairpin structure in an untranslated region between genes *F* and *G* (29). This location is analogous to that of the hairpin loop that serves as the unique origin for complementary strand synthesis for phage G4 (30, 31). The protein n′ recognition site is two-thirds of the distance around the circle from the origin of viral strand replication. It seems plausible that the start of the φX complementary strand might be made at the exposed protein n′ recognition site even before the viral circle is completed.

The start of viral strand synthesis by the purified RF → SS system is unequivocally at the unique cleavage site created by gene *A* protein and produces viral strand continuously. Despite observations of RF multiplication *in vivo* that have led others to propose that the starts of viral strands are multiple and discontinuous (32, 33), the evidence adduced from our *in vitro* system suggests to us that continuous synthesis of viral strands in intact cells may prove to be the preferred mechanism.

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