Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 40, Issue of October 1, pp. 28059–28062, 1999

Replication of Herpes Simplex Virus DNA*

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Herpes simplex virus type 1 (HSV-1)[1] is a member of the herpes group of viruses, the *Herpesviridiae*, which includes the important human pathogens herpes simplex virus type 2, cytomegalovirus, varicella zoster virus, Epstein-Barr virus, human herpesviruses 6 and 7, and Kaposi's associated herpesvirus, human herpesvirus 8 (reviewed in Ref. 1). Of these, HSV-1 has been the most extensively studied. The HSV-1 genome is a 153-kilobase linear duplex with 75 open reading frames. It consists of two unique regions, U_L (unique long), comprising 82% and the U_S (unique short) comprising 18%, flanked by the repeated regions *a*, *b,* and *c* (Fig. 1). As a consequence of recombination through the *a* sequences, the HSV-1 genome actually exists as an equimolar mixture of four isomeric forms generated by inversion of the U_L and U_S sequences relative to each other.

An interesting but poorly understood aspect of the HSV-1 life cycle, in fact, the life cycle of all the herpesviruses, is the ability to enter latency, a state in which no infectious progeny are produced and only limited transcription of viral genes occurs. Upon presentation of the appropriate stimulus, the latently infected cells enter a lytic phase with the resultant production of infectious viral particles (1).

In the lytic phase, following penetration of the nucleus, a temporally regulated program of viral gene expression mediated by RNA polymerase II begins. A virion protein, VP16, acts *in trans* together with the cellular octamer DNA-binding protein, Oct-1, to induce the α or *immediate early* genes, the first set of HSV-1 genes to be expressed. These serve as transactivators of β or *early* gene expression. Their synthesis peaks at 2–4 h, postinfection. Functional α proteins are required for the synthesis of all the virally encoded proteins. Synthesis of the β class of proteins reaches peak rates 5–7 h and is detectable as early as 3 h postinfection. The β proteins include the enzymes that are required for replication of the viral genome: a DNA polymerase, a single-strand DNA-binding protein, a primosome or helicase-primase, an origin-binding protein, and a set of enzymes involved in DNA repair and in deoxynucleotide metabolism. Homologues of these proteins are found in virtually all herpesviruses (1, 2).

Viral DNA synthesis begins shortly after the appearance of the β proteins and is detectable as early as 3 h and continues up to 15 h postinfection, with the bulk of the DNA synthesized during the latter part of the period. The temporal program of viral gene expression ends with the appearance of the γ or *late* proteins, which constitute the structural proteins of the virus (1).

Structure of Replicating DNA

As early as 0.5 h postinfection and in the absence of protein synthesis, viral DNA accumulates in the nucleus and adopts an "endless" configuration whose structure is consistent with circularization (1). Although the mechanism of genome circularization has not been established, it may involve a recombination event through the terminal repeat sequences. One clear advantage of this intermediate is that it eliminates the need for a specialized mechanism to replicate the termini.

Although there is no firm proof for the existence of *theta* replication intermediates that initiate at one or more of the HSV-1 origins *in vivo*, genome circularization is a prerequisite for viral replication, and replication requires either ori_S or ori_L and the action of a viral protein that possesses all the properties of an initiator protein (UL9 protein). These observations strongly suggest that HSV-1 DNA replication initiates by a *theta* mechanism and make it unlikely that other mechanisms, such as inter- or intramolecular recombination or transcription, all of which could potentially occur on linear DNA templates, are responsible for the initiation of HSV-1 DNA replication. At some point and by an as yet undefined mechanism, *theta* replication switches to the rolling circle mode, the predominant mode of HSV-1 DNA replication.

In the rolling circle mode, DNA replication generates long head-to-tail concatamers similar to the DNA replication products observed during the theta phase of bacteriophage λ replication (3). At late times during viral DNA replication, the products consist of branched networks of concatamers that may arise from recombination and invading DNA replication forks.

Origins of DNA Replication

The HSV-1 genome contains three *cis*-acting elements, which function as origins of DNA replication: one copy of ori_L, located between the diverging *UL29* and *UL30* genes within U_L, and two copies of ori_S, located within the *c* sequences (Fig. 2).

Both ori_S and ori_L contain large palindromes of 45 and 144 bp, respectively, that center around an A + T region of 18 and 20 bp, respectively. Flanking the A + T region are inverted repeats that are binding sites for the HSV-1-encoded origin-binding protein (UL9 protein).

It is unclear why the HSV-1 genome contains multiple origins that appear identical in every respect other than size and location. The existence of two copies of ori_S may be rationalized by its location in a repeated *c* region of the genome. However, neither copy of ori_S is essential because deletion of one or both copies of ori_S has no effect on viral DNA replication in cultured cells (1).

HSV-1 Gene Products Essential for Origin-specific DNA Replication

The development of a transient DNA replication assay in which origin-containing plasmids are replicated by transfected HSV-1 sequences that supply *trans*-acting factors led to major advances in the identification of the essential HSV-1 DNA replication genes (4). This analysis permitted identification of seven HSV-1 genes all located within the U_L segment of the genome that are necessary and sufficient for origin-specific DNA replication: *UL5, UL8, UL9, UL29, UL30, UL42,* and *UL52* (Fig. 1) (5, 6). These assignments were subsequently confirmed by demonstrating that mutations in these genes abrogate viral replication (7, 8). Although the function of the *UL29* (single-strand DNA-binding protein) and *UL30* (DNA polymerase) gene products had already been identified, the remaining five genes encoded proteins of unknown function. These genes were subsequently correlated with proteins identified by biochemical analyses. The functions of these gene products are summarized in Table I, which also depicts the conservation of DNA replication functions in a variety of organisms/systems.

Origin-binding Protein (UL9 Protein)—A search for proteins that bind to an HSV-1 origin led to the identification of an HSV-1-induced factor that could recognize sequences within ori_S (9). Further purification of this sequence-specific DNA binding activity led to the identification of an 83-kDa protein that bound to two inverted repeats that flank the A + T region, within ori_S, designated Boxes I and II (Fig. 2) (10). In ori_L the two Box I sites are flanked by homologous sequences designated Box III, which differ

* This minireview will be reprinted in the 1999 Minireview Compendium, which will be available in December, 1999. The work in the authors' laboratories is supported by National Institutes of Health Grants AI26538 (to I. R. L.) and AI38335 (to P. E. B.).
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[1] The abbreviations used are: HSV-1, herpes simplex virus type 1; bp, base pairs.

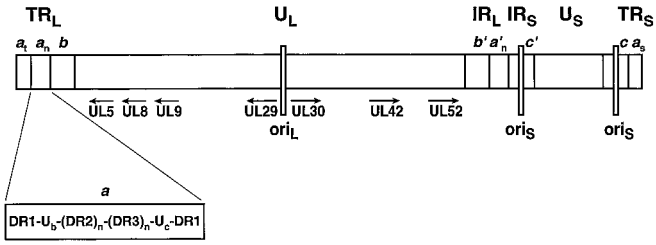

FIG. 1. **Structure of the herpes simplex virus type 1 genome.** A diagrammatic representation of the HSV-1 genome is shown. The positions of the *a*, *b*, and *c* repeats within the terminal repeats (TR_L and TR_S) and internal repeats (IR_L and IR_S) and the positions of the DNA replication origins (ori_L and ori_S) are as indicated. The position and direction of transcription of the seven essential DNA replication genes are indicated by the *arrows*. The *enlarged area* shows the composition of a unit length *a* sequence, consisting of direct repeats (*DR*) *1*, *2*, and *3* and unique (*U*) domains *b* and *c*. *t*, terminal; *n*, variable number of copies; *′*, inverted orientation; *L*, long; *S*, short. This drawing is not to scale; see text for details. (Reprinted with permission from Ref. 2.)

from Box I by only 1 residue but act as low affinity recognition sites for the UL9 protein. In ori_S there is only one copy of Box III, 5′ to Box I. Subsequently, it was demonstrated that the origin-binding protein is the product of the *UL9* gene (11). The *UL9* gene encodes an 851-amino acid polypeptide (UL9 protein) with a calculated mass of 94,246 Da (12). Amino acid sequence analysis of the UL9 protein led to the identification of conserved ATP binding and DNA helicase motifs (13, 14) that have been shown to be essential for viral replication (15, 16). The sequence-specific DNA binding activity of the UL9 protein resides in the C-terminal 317 amino acids (16). Expression of the 37-kDa C-terminal DNA-binding domain has a dominant negative effect on viral replication, presumably because of its ability to occupy the origin nonproductively (16, 17). Although the UL9 protein can bind to its recognition sequence as a homodimer, only one of the two monomers contacts the DNA (18). The dimerization of UL9 protein is mediated through the N-terminal part of the protein, presumably through a leucine zipper motif encompassing residues 150–171 (19, 20). The binding of UL9 protein to ori_S appears to be enhanced by nucleoside triphosphates (21).

In addition to its sequence-specific DNA binding activity, the UL9 protein possesses DNA-stimulated nucleoside triphosphatase and nonspecific 3′ → 5′-DNA helicase activities (22–25). ICP8, the HSV-1 single-strand DNA-binding protein, interacts with the extreme C terminus of the UL9 protein, greatly stimulating its DNA-dependent ATPase and DNA helicase activities (25). In the absence of ICP8 the UL9 protein is non-processive. ICP8 appears to stimulate the UL9 protein by preventing its dissociation from the DNA (25, 26).

The UL9 protein, together with ICP8, can unwind specifically Box I of ori_S if an appropriately oriented 3′ single-stranded tail of at least 18 nucleotides (the binding site size for ICP8; see below) is attached to Box I (27). This finding suggests that a complex consisting of the UL9 protein and ICP8 bound to appropriately oriented single-stranded DNA constitutes the specific helicase that can separate the two strands, permitting entry of the DNA replication machinery. The single-stranded DNA-binding site for ICP8 in ori_S may be provided by the A + T sequence that links Boxes I and II. Because of its relatively weak hydrogen bonding, this sequence may be easily unwound, either by the UL9 protein itself or as a consequence of the initiation of transcription at the promoters that flank the origin. This mode of unwinding of an HSV-1 origin by the UL9-protein-ICP8-single-stranded DNA complex differs from that seen with the SV40 T antigen, in which the T antigen itself is the origin-specific helicase and the SSB plays a purely passive role binding the single strands generated by helicase action and thereby preventing their reannealing (28).

Single-strand DNA-binding Protein, ICP8— ICP8 is the product of the *UL29* gene (29) and consists of 1196 amino acids with a calculated molecular mass of 128,342 Da.

ICP8 binds single-stranded DNA rapidly and cooperatively and with at least 5-fold greater affinity than double-stranded DNA (30, 31). Examination of ICP8 binding to single-stranded DNA, by electron microscopy and negative staining, showed regular protein-DNA filaments in which the DNA is held in an extended configuration (32, 33). Estimates of the DNA-binding site size for ICP8 fall in the range of one ICP8 to 12–22 nucleotides (34–36), values that are in agreement with a site size of 15–18 nucleotides estimated by electron microscopy (33).

Primosome (DNA Helicase-Primase)—The HSV-1-encoded primosome consists of three subunits with molecular masses of 98,710, 79,291, and 114,416 Da, the products of the *UL5*, *UL8*, and *UL52* genes, respectively (6).

The holoenzyme consists of a 1:1:1 association of the *UL5, UL8,* and *UL52* gene products, with a native molecular mass of ~270 kDa (37, 38). A subassembly that consists of the UL5 and UL52 subunits retains DNA-dependent ATPase, helicase, and primase activities and therefore constitutes the core enzyme (39, 40). In contrast, the UL8 protein lacks detectable enzymatic or DNA binding activities (40, 41). Under the appropriate conditions of Mg^{2+} concentration and ionic strength the helicase activity of the helicase-primase can unwind 60–65 bp/s, a value consistent with the rate of 50 bp/s estimated for the rate of fork movement *in vivo* during replication of pseudorabies virus, another herpesvirus (42).

Residues 610–636 of the UL52 protein contain a proposed divalent metal binding motif that is conserved in DNA polymerases and primases (43, 44). Site-directed mutagenesis of this motif inactivates the primase activity of the mutant holoenzyme *in vitro* and abolishes origin-specific DNA replication *in vivo*, indicating that it comprises the primase active site. The products of primase action are oligoribonucleotides that are 6–13 bases in length (45, 46). With single-stranded ϕX174 DNA as template, the primase exhibits sequence specificity. The preferred template sequence was mapped to 3′-AGCCCTCCCA, directing the synthesis of a 10-mer oligoribonucleotide, starting at the underlined C residue (48).

The UL8 protein stimulates primer synthesis by the UL5/52 subassembly (47–49). Thus, although UL8 protein has no effect on the $K_{m(DNA)}$ (15 μM nucleotides) of the subassembly, it increases the rate of primer synthesis approximately 3-fold (47). The UL8 protein is also required for optimal DNA helicase and DNA-dependent ATPase activities of the enzyme in the presence of ICP8-coated DNA templates (49, 50). It therefore resembles the helicase/primase loading factors seen in other systems (see Table I). The *E. coli* single-strand DNA-binding protein cannot substitute for ICP8, suggesting a specific interaction between ICP8 and the UL8 protein. Surface plasmon resonance measurements did, in fact, demonstrate an interaction between ICP8 and the U5/52/8 heterotrimer but not with the UL5/52 subassembly or the UL8 protein alone (49).

DNA Polymerase—The HSV-1 DNA polymerase has been intensively studied, both as a model eukaryotic DNA polymerase and as a target for antiviral drugs. It is a 1235-amino acid, 136,413-Da protein that is encoded by the *UL30* gene (29, 51–53). The UL30 protein exhibits significant sequence similarity to other viral and cellular DNA polymerases including human DNA polymerase α-primase, *Saccharomyces cerevisiae* DNA polymerase δ, *E. coli* DNA polymerase I, and the bacteriophage T4 DNA polymerase (54, 55).

The HSV-1 DNA polymerase purified from virus-infected cells exists as a heterodimer in which the UL30 protein is associated with a 65-kDa protein (56–58). The 65-kDa protein is the product of the *UL42* gene which encodes a 488-amino acid, 51,154-Da phosphoprotein that possesses double-strand DNA binding activity (6, 59). The interaction between the UL30 and UL42 proteins occurs with an association constant of 1×10^8 M^{-1} (60) and has been shown to be essential for viral replication (61).

The HSV-1 DNA polymerase possesses 3′ → 5′-exonuclease activity that is intrinsic to the UL30 subunit (62, 63). The exonuclease is active under conditions that are optimal for deoxynucleotide polymerization and can act on a variety of DNA substrates including gapped DNA, DNA hairpin structures, and single-stranded oligodeoxyribonucleotides (63). In the absence of dNTPs, the 3′ → 5′-exonuclease is twice as active at removing 3′-unpaired deoxynucleotides as paired deoxynucleotides. In the presence of all four dNTPs, there is no detectable 3′ → 5′-exonuclease on paired deoxynucleotides, presumably because of continuous polymerization. In contrast unpaired deoxynucleotides are completely removed (63). Consequently, the 3′ → 5′-exonuclease serves as a proofreading activity to ensure high fidelity of DNA replication. In addition to its 3′ → 5′-exonuclease, the HSV-1 DNA polymerase has a RNase H activity that presumably serves to remove RNA primers during the processing of Okazaki fragments (58).

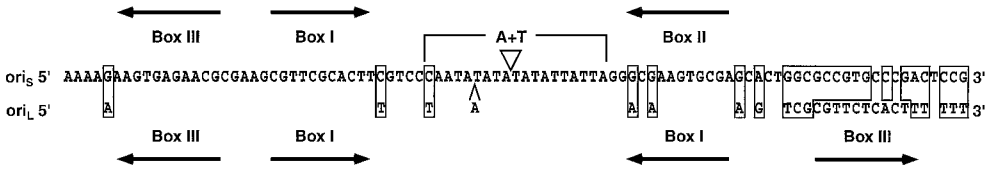

FIG. 2. **Structure of the herpes simplex virus type 1 origins of DNA replication, ori_S and ori_L.** The DNA sequence of the minimal ori_S is shown. *Boxed bases* indicate sequence differences between ori_S and ori_L. The *arrows* depict the relative orientation of the UL9 protein recognition sites, Boxes I, II, and III. The *triangle* indicates the center of symmetry: *A + T*, sequence. (Reprinted with permission from Ref. 2.)

TABLE I
Analogous DNA replication functions in bacteriophages, bacteria, animal viruses and eukaryotes

	E. coli/phage λ	Phage T4	Phage T7	HSV-1	*S. cerevisiae*[b]	SV40/*Homo sapiens*
Origin-binding protein	DnaA/O protein	None	None	UL9 protein	ORC	T antigen/?
Helicase-primase loading protein	DnaC/P protein	gp59	None	UL8 protein	Cdc6 protein?	T antigen/?
Replicative helicase	DnaB	gp41	gp4	UL5/52 proteins	MCM proteins?	T antigen/?
Primase	DnaG	gp61	gp5	UL5/52 proteins	DNA pol α-primase	DNA pol α-primase
DNA polymerase	DNA pol III[a]	gp43	gp5	UL30/42 proteins	DNA pol δ/ε?	DNA pol δ/DNA pol δ/ε?
Proofreading exonuclease	ε subunit	gp43	gp5	UL30 protein	DNA pol δ/ε?	DNA pol δ/DNA pol δ/ε?
DNA polymerase clamp	β subunit	gp45	*E. coli* thioredoxin	UL42 protein	PCNA	PCNA
DNA polymerase clamp loader	γ complex	gp44/62	None	None	RF-C	RF-C
Single-strand DNA-binding protein	SSB	gp32	gp2.5	ICP8	RP-A	RP-A
RNase H	RNase H	gp33.2	gp6	UL30 protein	RNase H1	RNase H1
5′ → 3′-Exonuclease	DNA pol I	gp33.2	gp6	UL12 protein?	FEN-1	FEN-1

[a] pol, polymerase.
[b] ORC, origin recognition complex; MCM, maintenance of minichromosome; PCNA, proliferating cell nuclear antigen; RF, replication factor; FEN, flap endonuclease; RP, replication protein.

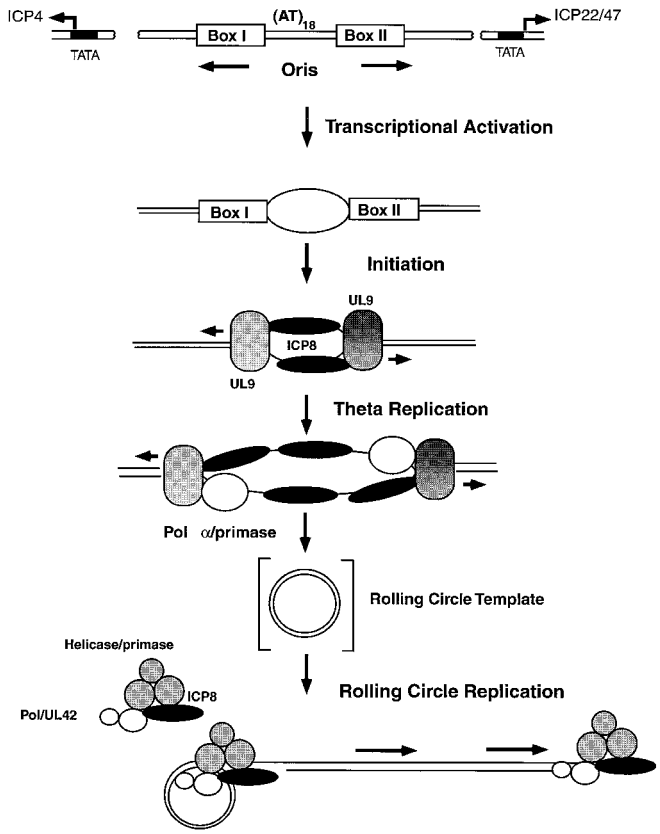

FIG. 3. **Model for the bipartite replication of the HSV-1 genome in which an initial transient phase of *theta* replication is followed by a rolling circle mode, the predominant mode of HSV-1 DNA replication.**

The UL42 protein acts to increase the processivity of the UL30 DNA polymerase (36, 64, 65). It is therefore a functional homologue of the DNA polymerase processivity enhancing factors, which include the β subunit of *E. coli* DNA polymerase III holoenzyme, the eukaryotic proliferating cell nuclear antigen, and bacteriophage T4 gene 45 protein (28, 66) (see Table I). These proteins form a ring that encircles the DNA duplex and interact with their cognate DNA polymerase to prevent dissociation of the enzyme from the primer-template, thereby ensuring high processivity of deoxynucleotide polymerization. However, these DNA polymerase clamps lack DNA binding activity, and their assembly on the DNA is mediated by another set of proteins, the clamp loaders. In contrast, the HSV-1 DNA polymerase is tethered to the DNA by the double-strand DNA binding activity of the UL42 protein itself. This model is supported by the finding that stimulation of DNA polymerase activity requires the DNA binding activity of the UL42 protein (64, 65).

Numerous nucleotide analogues (9-(2-hydroxyethoxymethyl) guanosine 5′-triphosphate (acyclovir triphosphate), 9-β-D-arabinofuranosyladenosine 5′-triphosphate (araATP), aphidicolin, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine 5′-triphosphate) and pyrophosphate analogues (phosphonoacetate, phosphonoformate, oxalate) have been examined for their ability to inhibit viral replication *in vivo* and to inhibit selectively the HSV-1 DNA polymerase *in vitro*. This has led to the development of several antiviral drugs, notably gancyclovir and acyclovir, which are of significant therapeutic value (52).

In addition to ICP8, the DNA polymerase-UL42 complex, the UL9 protein, and the DNA helicase-primase, all of which are essential for HSV-1 DNA replication, the HSV-1 genome encodes a set of enzymes whose function is not required for its replication in cultured cells. They undoubtedly serve some function in the viral life cycle. These include an alkaline endo-exonuclease that exhibits 5′ → 3′-exonuclease activity (UL12 protein) and may thus participate in the completion of lagging-strand DNA synthesis, a uracil DNA glycosylase, a deoxyuridine triphosphatase, a thymidine kinase, and a ribonucleotide reductase (2).

Attempts to Reconstitute HSV-1 DNA Replication in Vitro

As described previously, studies of HSV-1 DNA replication *in vivo* have demonstrated that the linear 153-kilobase pair genome circularizes shortly after infection of susceptible host cells and then enters a rolling circle mode of DNA replication generating branched concatameric DNA, which is then cleaved and packaged as unit-length molecules. The existence of specific origins of replication ori_S or ori_L as well as a protein (the UL9 protein) with helicase activity that binds the origins with high affinity suggests that a theta mode of DNA replication precedes the rolling circle phase (Fig. 3). A bipartite mode of DNA replication of this kind has been observed for bacteriophage λ (3). Although, as noted above, rolling circle replication of the HSV-1 genome as well as of plasmids into which an HSV-1 origin has been inserted has been observed *in vivo*, there is as yet no compelling evidence *in vivo* for a theta mode of replication.

Numerous attempts have been made to observe origin (ori_S or ori_L)-dependent DNA replication *in vitro*. None has thus far been successful.

In contrast to the inability to reconstitute the theta model of

HSV-1 DNA replication, rolling circle replication promoted by extracts of HSV-1-infected cells has been achieved. The first demonstration of rolling circle replication made use of an artificial replication fork consisting of M13 single-stranded DNA to which a complementary oligonucleotide with an unpaired 5′ single-stranded tail had been annealed. Rolling circle replication was observed both with crude extracts of HSV-1-infected cells and with a partially purified fraction containing the HSV-1 DNA polymerase and the UL5 and UL52 subunits of the DNA helicase-primase. ICP8 stimulated the reaction but was not essential (67).

In a second approach, a high molecular mass complex ($M_r > 10^6$) was isolated by gel filtration and ion-exchange chromatography from extracts of insect (Sf9) cells that had been multiply infected with baculoviruses recombinant for the seven HSV-1 genes that are required for its replication. This complex, which consisted of the HSV-1 DNA polymerase, ICP8, and DNA helicase-primase, could promote the rolling circle replication of circular plasmid templates to generate concatameric DNA molecules up to four times unit length as judged by gel electrophoresis and electron microscopy. Neither the UL9 protein nor an HSV-1 origin was required, and in fact, the presence of ori_S within the plasmid inhibited DNA replication in the presence, but not the absence, of the UL9 protein (68).

Whole-cell extracts of HSV-1-infected human cells (293 cells) can also promote the rolling circle replication of plasmid templates in a reaction that closely resembles that observed with the high molecular weight complex isolated from the baculovirus-infected Sf9 cells (69). Preliminary evidence suggests that a similar enzyme complex is present in the HSV-1-infected 293 cells. Although the rate of fork movement in these reactions has not been accurately determined, it appeared to approach the *in vivo* rate cited above. However, the efficiency of the reaction is very low (1–2% of the template molecules replicated) for both the complex obtained from the baculovirus-infected Sf9 cells and the extract of HSV-1-infected 293 cells. The form of the plasmid DNA that serves as the template for rolling circle replication is not known, and the small proportion of the template molecules replicated may reflect the low level of the appropriate template that is generated by either the enzyme complex or the crude extract. Possibly a recombinational event analogous to that which appears to be involved in the switch from theta to rolling circle replication in bacteriophage λ may be required (3).

In summary, HSV-1 is a large (153 kilobase pairs), double-stranded DNA virus whose genome contains multiple, redundant origins of replication. HSV-1 encodes a repertoire of proteins that, with the exception of a DNA ligase and topoisomerase, should suffice to initiate and sustain DNA replication. The current view is that following circularization of the linear HSV-1 genome, replication proceeds initially by origin-dependent *theta* followed by origin-independent rolling circle DNA replication. A host DNA polymerase, DNA polymerase α-primase, may function during the *theta* phase (70) (Fig. 3). Finally, the functions of the HSV-1 DNA replication proteins resemble those found in other organisms, further indicating strong conservation in the mechanisms of DNA replication.

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