The in Vitro Replication of DNA Containing the SV40 Origin

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The SV40 genome contains a single origin of DNA replication within a small (5243 base pairs) covalently closed double-stranded DNA genome and is organized into mini-chromosomes containing histones derived from the host cell. With the exception of the virus-encoded large T antigen, all the proteins required for SV40 replication are supplied by the host cell. Thus, many of the essential host proteins for SV40 DNA replication probably play a similar role in cellular DNA replication. The establishment of an in vitro SV40 DNA replication system by Li and Kelly (1) has been pivotal in allowing the characterization of the host proteins which play a role in SV40 replication. This review will focus on the replication system by Li and Kelly (1) has been pivotal in allowing the characterization of the host proteins which play a role in SV40 replication. This review will focus on the pathway of SV40 DNA replication and the proteins involved in the DNA synthesis reactions. Other reviews of eukaryotic DNA replication have also appeared recently (2-5).

A Model of the DNA Replication Fork during SV40 DNA Synthesis

In order to introduce the proteins involved in carrying out DNA synthesis, a model of the replication fork is presented in Fig. 1, and a summary of their properties is presented in Table I. A number of ATP-dependent pre-DNA synthetic reactions at the core origin of replication, carried out by T antigen, summarized by Borowiec et al. (6), lead to DNA unwinding in the presence of a single-stranded DNA binding protein and topoisomerase I. At some point prior to the extensive unwinding of the duplex from the core origin, T antigen may interact with the DNA pol α-DNA primase complex which initiates RNA-primed DNA synthesis (7, 8).

As shown in Fig. 1, DNA pol α-primase, on the lagging strand, synthesizes short RNA-primed DNA fragments in the presence of the three-subunit human SSB (HSSB).1 Joining of these “Okazaki fragments” to form a completed daughter strand requires the action of a 5’→3’-exonuclease and RNase H to remove the RNA primers, filling in of the gaps by a polymerase, and sealing the nicks by DNA ligase (9). Finally, topoisomerase II can decatenate the daughter molecules (9, 10).

DNA pol δ is shown carrying out elongation of the leading daughter strand in the presence of HSSB and in conjunction with the protein factors PCNA and activator 1 (Al) (11), also identified as RF-C (12).

Since DNA polymerases cannot initiate DNA chains de novo (13), DNA primase is required to synthesize small oligonucleotide primers. In a coupled reaction, these primers are immediately extended by the DNA pol α complexed to primase. In contrast, pol δ lacks DNA primase activity and extends RNA primer ends poorly. Thus, the pol α-DNA primase complex is responsible for the synthesis of the first DNA segment, which could be considered the first lagging strand Okazaki fragment but is subsequently utilized as the primer for the initiation of leading strand synthesis by pol δ. In the presence of PCNA and Al, pol δ elongates the leading strand, while pol α-primase continues synthesis of the lagging strand as T antigen unwinds the duplex.

Role of the Single-stranded DNA Binding Protein

No other SSB examined can efficiently replace the three-subunit SSB (Table I) isolated from human cells (HSSB) in the SV40 replication reaction (14, 15, 56). A similar three-subunit SSB from yeast partially replaces the HSSB in replication (16). This is in contrast to the T antigen-mediated unwinding reaction for which SSBRs isolated from Escherichia coli, adenovirus, and herpes simplex virus all substituted for HSSB (17). The SSBRs that support the elongation of primed DNA templates by both DNA pol α and pol δ are the HSSB and yeast SSB.2 Pol α was inhibited by all SSBs tested with the exception of the three subunit SSBRs (human and yeast), while pol δ was active in the presence of all SSBs tested. Furthermore, monoclonal antibodies directed against either the human 70-kDa subunit, which binds DNA, or the human 34-kDa subunit block the replication reaction, suggesting that both subunits are essential (18).

The sequence of the human 34-kDa subunit has been determined (19). In yeast and human cells, the state of phosphorylation of this subunit appears to be cell cycle-regulated (16, 20). The role of phosphorylation of the 34-kDa subunit in replication is unknown.

Involvement of PCNA

PCNA is present in the cell nucleus at locations that correspond to the sites of DNA synthesis, which suggested that it plays a role in DNA synthesis (21, 22). This was further supported when SV40 DNA replication in vitro was shown to require PCNA (23, 24). PCNA was required for synthesis of the leading DNA strand, and in its absence only DNA products from the lagging strand template, 150 nucleotides long, accumulated (23, 25, 26). In addition, an accessory factor for DNA pol δ (27) was found to be identical to PCNA (28, 29). Based on these results, it was suggested that SV40 DNA synthesis was carried out on the lagging strand by DNA pol α-primase complex, and on the leading strand by PCNA and DNA pol δ (30, 31).

Monopolymerase and Dipolymerase Systems

After initiation of the two nascent strands by DNA primase and limited elongation by DNA pol α, the switch to leading strand synthesis by pol δ occurs. We have called this the dipolymerase phase of the replication reaction. In the SV40 replication system, Tsurimoto and Stillman (12, 32) have shown that PCNA and a multi-subunit protein, RF-C, are both essential for leading strand synthesis. In the absence of either one, or both, of these proteins SV40 DNA replication

1The abbreviations used are: SSB, single-stranded binding protein; HSSB, human single-stranded binding protein; PCNA, proliferating cell nuclear antigen; Al, activator 1; pol, polymerase; RF, replicative factor.
2S.-H. Lee, A. Kwong, Z.-Q. Pan, and J. Hurwitz, J. Biol. Chem., submitted for publication.
3S.-H. Lee, unpublished results.
was reduced 3–4-fold. The synthesized DNA was derived from the lagging strand, and the length of these products resembled Okazaki-sized fragments. These results indicate that both PCNA and RF-C promote leading strand synthesis.

Prior to these findings, Wobbe et al. (14) demonstrated that extensive bidirectional replication of SV40 origin-containing (ori⁺) DNA could be achieved by the combined action of T antigen, HSSB, topoisomerase I, and the pol α-primase complex (the monopolymerase system). The monopolymerase system supported the synthesis of Okazaki-sized fragments, arising from lagging strand templates, and longer products, derived from the leading strand (9).

The laboratories of Stillman and Kelly (24, 26) reported that replication reactions carried out with SV40 ori⁺ DNA, T antigen, topoisomerase I, HSSB, and pol α-primase complex predominantly synthesized small Okazaki fragments, arising from the lagging strand template. This may be explained by the finding that, in the monopolymerase system, the efficient synthesis of long DNA products is dependent on high levels of pol α-primase (0.2–0.4 unit), while smaller DNA fragments are predominantly synthesized in the presence of lower levels (0.02–0.04 unit) of pol α-primase (11).

The monopolymerase system was unaffected by PCNA or the addition of antibodies that neutralized PCNA. However, DNA synthesis using crude extracts of HeLa cells was inhibited more than 90% by neutralizing antibodies to PCNA. This inhibition was substantially reversed by PCNA (33). These results prompted our laboratory to further fractionate crude extracts, selecting for DNA synthesis dependent on both PCNA and T antigen. This resulted in the isolation of three protein fractions which were called elongation inhibitor and activators I and II. These three protein factors were subsequently identified as poly(ADP-ribose) polymerase, Al (a multisubunit protein which appears to be identical to RF-C), and PCNA-dependent pol δ, respectively (11).² The monopolymerase system was blocked by the binding of poly(ADP-ribose) polymerase at the ends of DNA chains, resulting in the accumulation of small Okazaki fragments which arose from the lagging strand template. However, in the presence of Al, ATP, and PCNA, poly(ADP-ribose) polymerase-blocked ends were rapidly elongated by pol δ.

In the absence of poly(ADP-ribose) polymerase, DNA synthesis using the dipolymerase system is dependent on the amount of pol α-DNA primase added. In the presence of low levels of pol α-primase (0.02 unit and below), DNA synthesis with the dipolymerase system was totally dependent on Al, PCNA, and pol δ. Under these conditions, long DNA products were formed, and virtually no Okazaki fragments were detected. This is probably due to the efficiency with which the pol δ-PCNA-A1 system can bind to and elongate low levels of primer ends. Thus, the majority of the labeled products that accumulated were due to leading strand synthesis. In the presence of high levels of pol α-primase (0.2–0.4 unit), which can synthesize both short and long products even in the absence of the pol δ system, the effects of pol δ, PCNA, and A1 on DNA synthesis were less apparent. The addition of poly(ADP-ribose) polymerase increased the rate of synthesis of long DNA products, and this effect was especially evident after incubation periods of 5 or 10 min. However, based on these findings, poly(ADP-ribose) polymerase is not essential for leading strand synthesis.

![Fig. 1. Model of SV40 DNA replication fork and the positioning of various proteins on the DNA.](image)

| Protein                                      | Molecular mass | Function of protein                                                                 |
|----------------------------------------------|----------------|-------------------------------------------------------------------------------------|
| SV40 T antigen                               | 82 kDa         | Origin binding; DNA helicase; interaction with pol α-primase complex                 |
| HSSB (RFA) (RPA)                             | 70, 34, 11     | Only SSB that supports SV40 replication; stimulates pol α and pol 4–70-kDa subunit binds DNA |
| DNA pol α-primase complex                    | 180, 70, 55, 48| Essential for SV40 synthesis; primase starts chains on both strands; species specific; 180-kDa catalytic subunit, 55 + 48-kDa subunits constitute primase |
| Topoisomerase I                              | 100 kDa        | Relieves positive superhelicity; essential in absence of topoisomerase II            |
| Topoisomerase II                             | 172 kDa        | Relieves positive superhelicity; essential in absence of topoisomerase I             |
| PCNA                                         | 37 kDa         | Essential for T antigen; acts as auxiliary factor in pol 4 elongation reaction       |
| Pol δ                                        | 125 kDa        | Involved in leading strand synthesis; contains 3′–5′-exonuclease                      |
| A1 (RF-C)                                    | 145, 40, 38, 37, 36,5 | Binds to primer-template; interacts with PCNA; acts as auxiliary factor in pol δ elongation reaction; contains DNA-dependent ATPase; increases affinity of pol δ for primer ends; reduces PCNA requirement in pol δ reaction |
| Poly(ADP-ribose) polymerase                  | 120 kDa        | Binds to ends of DNA chains; blocks elongation of 3′-OH ends by pol α; is reversed by PCNA, A1, and pol δ |
| RNase H                                      | 49, 39 kDa     | Stimulates RFI production; aids in removal of 5′-ribonucleotides                      |
| 5′→3′-exonuclease                            | 44 kDa         | Required for RFI production; removes 5′-ribonucleotides                               |
| DNA ligase I                                 | 102 kDa        | Required for formation of closed circular duplex products; seals nicks in daughter DNA strands |

² Isolated as 74-kDa dimer. ³ Isolated as 160-kDa dimer.
The Role of Activator 1 in DNA Synthesis

The multi-subunit A1 selectively bound to primer ends and increased the affinity of pol 6 for primer ends about 10-fold and decreased the amount of PCNA required for pol 6 as much as 100-fold. A1 contains an intrinsic DNA-dependent ATPase activity. However, its binding to primer ends was stimulated only 2-fold by ATP. The DNA-dependent ATPase activity of A1 (RF-C) could be stimulated 3–4-fold by PCNA (34), and the further addition of HSSB stimulated the DNA-dependent ATPase activity of A1 about 2-fold.2

The action of A1 and PCNA resembles the role played by the accessory proteins that participate in the elongation of primed templates by the T4 DNA pol (35, 36) and the E. coli DNA pol III systems (13). In the T4 (37–40), E. coli (13, 41–43), and human systems, the action of the accessory proteins requires ATP hydrolysis. The effects of the accessory proteins, A1 and PCNA, in the pol 6 system also require ATP. As in the prokaryotic systems, there is a precise order in the formation of the elongation complex with primed templates. The stepwise addition of the accessory proteins in these reactions is summarized in Fig. 2. In the E. coli system, the γδ subunits can bind to primed DNA, and the product can be filtered through a sizing column to separate free protein from the DNA-protein complex. In the next step, the addition of dα2N to the complex required the presence of ATP. After gel filtration, the complex supported elongation after the addition of pol III.

In the T4 phase system, the T4 DNA pol must be added to the 44/62-DNA complex prior to the addition of the gene 45 product (40). The ATP-dependent reaction is involved in the binding of the gene 45 product to the 44/62-43 (T4 DNA pol) DNA complex.

The order of addition of the accessory proteins that support the elongation of primed templates in the pol 6 system more closely resembles the E. coli system than the T4 system. The ATP-dependent step, involving the binding of PCNA to the A1-primed DNA complex, precedes the binding of pol 6.

Functionally, the pol 6 accessory proteins resemble auxiliary proteins of both prokaryotic systems. The 44/62, γδ, and A1 (RF-C) proteins are all multi-subunit DNA-dependent ATPases. The gene 45, dα2N, and PCNA proteins all require ATP for their association with other corresponding proteins.

Processivity in the Elongation Reaction

The T4 and E. coli prokaryotic polymerase accessory proteins convert their respective DNA polymerase from an enzyme that acts with moderate processivity to an enzyme that acts processively over long stretches of DNA. It has been postulated that accessory proteins act as clamps, increasing the association of the polymerase to primer-template (44).

In the pol 6-catalyzed elongation reaction, PCNA is essential for DNA synthesis, and the addition of increasing levels of A1 stimulated this reaction. However, the size of products was unaffected by the increased concentrations of A1. Rather, the level of nucleotide incorporation detected in the presence of PCNA and pol 6 alone was low, and the amount of nucleotide incorporated was markedly increased in the presence of A1. This suggests that while PCNA may be acting as a protein clamp to increase processivity, the role of A1 is to bring about the binding of pol 6 to the primer terminus, presumably through its interaction with PCNA bound to A1. It is possible that additional factor(s) will be found that will contribute to the processivity of pol 6.

Initiation of SV40 DNA Replication

The presynthetic reactions, mediated by T antigen and leading to the unwinding of the DNA duplex, are essential for the initiation of DNA synthesis. Experiments carried out with crude HeLa cell extracts, as well as with purified proteins (T antigen, DNA polymerase I, HSSB, and ATP), reveal a 10–15 min lag phase before unwound DNA accumulates (3, 45, 56). With purified proteins, up to 70% of origin-containing duplex DNA can be unwound (18).

Bullock et al. (46) examined the initiation reaction using crude extracts of HeLa cells. Precipitation of crude extracts with T antigen, ATP, and SV40 origin-containing DNA resulted in the accumulation of unwound DNA irrespective of the length of preincubation, pulse labeling, followed by a chase with unlabeled dNTPs, indicated that DNA regions neighboring and including the origin region served as the initial templates. This indicates that the preinitiation complex was fixed near the origin. Since there appears to be an interaction between T antigen and DNA primase-pol α complex, as well as species specificity in this interaction (47), it is possible that T antigen unwinding and the positioning of pol α-primase complex on the DNA are linked. Other factors that might be responsible for this positioning of the preinitiation complex are unknown.

Lagging Strand Model in SV40 Replication

Models to explain the variation in size of lagging strand products have been proposed in the T4 replication system (40, 44, 48, 49) which we find attractive for a number of reasons. In these models, as the T4 pol and accessory protein complex elongates primers on the lagging strand it remains bound to the primase-helicase complex, which acts to unwind the duplex at the replication fork. They propose that the release of the polymerase and accessory proteins from the 3'-OH end of the completed Okazaki fragment signals the initiation of a new primer by the primase-helicase in the complex. Studies with forked templates showed that the size of the Okazaki fragments increased in the presence of low concentrations of primase and polymerase (49). Similar effects have been observed in the SV40 monoplymerase and dipolymerase replication systems.

The model of Richardson et al. (40), applied to the SV40 system (Fig. 3), suggests the following features. The movement of T antigen governs the rate of chain growth on both leading and lagging strands. As the T antigen unwinds the duplex, it generates a loop on the lagging strand which is sequestered by the HSSB. In this model, the pol α-primase complex which is bound to T antigen synthesizes a short oligoribonucleotide immediately behind the T antigen on the single-stranded DNA which is devoid of HSSB. The RNA primer synthesized just behind the T antigen is then elongated.
by pol α, which traverses through the HSSR. As the elongation reaction proceeds, the unwinding activity of T antigen generates a single-stranded loop of DNA. The pol α-catalyzed elongation reaction continues until the new 3’-end becomes juxtaposed behind the preceding Okazaki fragment, which signals the release of pol α. This activates the primase to initiate the primer for the next Okazaki fragment formation.

In this model, synthesis on the leading strand is catalyzed by the pol δ system and is limited by the migration rate of T antigen and its generation of the leading strand template. This model predicts that the rate of lagging strand synthesis and T antigen unwinding govern the size of Okazaki fragments. Since low concentrations of pol α-primase yield longer replication systems, the length of the single-stranded loop on high levels of pol cu-primase complex, its association with T antigen, pol cu-primase traverses these loops, resulting in longer Okazaki chains. After being reassociated with T antigen, pol α-primase traverses these loops, resulting in longer Okazaki chains.

**Perspectives on the SV40 Replication System**

As recently reviewed by Prives (50), phosphorylation and dephosphorylation of T antigen plays a critical role in controlling the activity of T antigen. The origin binding proteins λ0 (52), dnaA (51), and T antigen are all required in high concentrations for activation of their well defined origin sequence. However, a specific eukaryotic origin and a T antigen equivalent have remained elusive. The discovery that structure rather than specific sequences in DNA govern primate origins may be important in defining eukaryotic origins. Thus, the identification of the cellular equivalent of T antigen may require the identification of an origin structure-specific binding protein rather than an origin sequence-specific binding protein.
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