An inhibitor of the in vitro elongation reaction of simian virus 40 DNA replication is overcome by proliferating-cell nuclear antigen

(DNA polymerases/leading and lagging strands/regulation of DNA synthesis)

SUK-HEE LEE, YUKIO ISHIMI, MARK K. KENNY, PETER BULLOCK, FRANK B. DEAN, AND JERARD HURWITZ

Graduate Program in Molecular Biology, Sloan-Kettering Cancer Center, New York, NY 10021

Contributed by Jerard Hurwitz, September 13, 1988

ABSTRACT The replication of simian virus 40 (SV40) origin-containing DNA has been reconstituted by using SV40 large tumor (T) antigen and cellular proteins purified from HeLa cells. This replication reaction is unaffected by proliferating-cell nuclear antigen (PCNA). In contrast, PCNA has been reported to stimulate SV40 DNA synthesis carried out with crude fractions [Prellich, G., Kostura, M., Marshak, D. R., Mathews, M. B. & Stillman, B. (1987) Nature (London) 326, 471–475]. This difference is caused by the presence of a protein in crude fractions that inhibits the elongation of nascent DNA chains during replication. In the presence of PCNA, crude fractions containing this elongation inhibition factor can extend DNA chains. We describe the partial purification of this inhibitor and show that its addition limited SV40 DNA replication to the synthesis of short chains, an effect reversed by the addition of PCNA. However, the reversal of the inhibition by PCNA in the SV40 system required additional protein fractions distinct from PCNA and the enzymes constituting the purified system. These results suggest that the PCNA-mediated effect on SV40 DNA replication may be indirect. Such an interplay between negative and positive regulatory functions including PCNA may contribute to the control of DNA synthesis characteristic of the eukaryotic cell cycle.

The replication of DNA containing the simian virus 40 (SV40) origin of replication can be carried out in vitro (1–3). Investigation of the proteins involved in this process has revealed required roles for DNA polymerase α and DNA primase (4), topoisomerases I and II (5), and a single-stranded-DNA-binding protein (SSB) purified from human cells (6–8). We have reported the reconstitution of a SV40 DNA replication system utilizing purified SV40 large tumor (T) antigen, HeLa SSB, topoisomerase I or II, and DNA polymerase α–primase complex (6), which supports both leading- and lagging-strand synthesis (9). Furthermore, closed circular duplex DNA products were formed when these proteins were supplemented with purified RNase H and a 5′ → 3′ exonuclease, which combine to remove RNA primers, and DNA ligase (9). In contrast, replication of SV40 origin-containing (ori+) DNA was recently shown to require an additional protein, proliferating-cell nuclear antigen (PCNA), when the reaction was carried out with partially purified fractions. PCNA was required for the synthesis of the leading DNA strand, and in its absence only low molecular weight DNA products (150 nucleotides long) accumulated (10, 11).

PCNA was identified previously as a protein that interacts with the sera of some patients with systemic lupus erythematosus (12, 13). It is detected in the nuclei of proliferating cells but not of quiescent cells, and it is identical to cyclin (14), a protein synthesized preferentially during the S phase of the cell cycle (15–18). In addition, PCNA was demonstrated by immunofluorescence to reside only in the cell nucleus and to increase during S phase (17, 19, 20). However, PCNA is quite stable in the cell, and its level remains fairly constant throughout the cell cycle (17, 19–21). These results led to the suggestion that PCNA plays some role in DNA replication (17, 20). This suggestion was further supported when a protein isolated as an accessory factor for DNA polymerase δ (23) was shown to be identical to PCNA (18, 24, 25), and with the demonstration of a role for PCNA in SV40 DNA replication (10, 11, 25).

Here we show that the synthesis of SV40 DNA with purified proteins is unaffected by PCNA or by antibodies against PCNA. To reconcile these findings with the observations described above, we investigated the nature of the PCNA effect in crude fractions of HeLa cell extracts. We detected a protein that inhibits the elongation phase of SV40 DNA replication. In addition, this inhibition factor blocks a number of reactions that occur at DNA ends, such as the action of DNA ligase and both 5′ → 3′ and 3′ → 5′ exonucleases. The inhibition of SV40 replication can be overcome by the addition of PCNA plus other protein fractions, which in the absence of the elongation inhibitor have no effect on the in vitro replication of SV40 DNA. These results suggest that the stimulation of SV40 DNA replication by PCNA is indirect, manifesting itself only in the presence of the inhibitor.

MATERIALS AND METHODS

Preparation of HeLa Extracts, DNA, T Antigen, PCNA, Anti-PCNA Serum, and Purified Enzymes. Cytosolic extracts of HeLa cells, SV40 ori+ DNA (pSV01ΔEP), SV40 T antigen, immunopurified DNA polymerase α–primase complex (1.2 × 10^6 and 4.1 × 10^6 units/mg of protein, respectively), topoisomerase I (6 × 10^6 units/mg), HeLa SSB (630 units/mg), 5′ → 3′ exonuclease (1.4 × 10^5 units/mg), and DNA ligase (0.8 unit/mg) were prepared as described (9). PCNA (5.2 × 10^7 units/mg) was purified to near homogeneity by a modification of the procedure of Prellich et al. (11, 25); a linear gradient of NaCl (0.175–0.6 M) was used in the DEAE-cellulose step, and hydroxylapatite (Bio-Rad) was substituted for the Q-Sepharose column. The source of the antibodies against PCNA was a human lupus serum (AK), which was generously supplied by Eng M. Tan. This serum was shown by Tan and coworkers to be relatively monospecific for PCNA (13, 26) and it inhibited the ability of PCNA to act as a DNA polymerase δ auxiliary protein (24).

Assay and Fractionation of the Elongation Inhibitor. The inhibitor was measured by the SV40 replication assay.

Abbreviations: SV40, simian virus 40; SV40 T antigen, SV40-encoded large tumor antigen; SSB, single-stranded-DNA-binding protein; PC, phoshocellulose; dsDNA, double-stranded DNA; PCNA, proliferating-cell nuclear antigen.
Reaction mixtures (40 μl) contained 6 μg (protein) of the fraction eluted from phosphocellulose by 0.3 M NaCl (0.3 M PC fraction, prepared as described below); 0.6 μg of SV40 T antigen; 0.8 μg of HeLa SSB; 105 units of topoisomerase I; 16 μg of bovine serum albumin; 1.0 μg of creatine kinase; 40 mM creatine phosphate (di-Tris salt, pH 7.7); 7 mM MgCl2; 0.5 mM dithiothreitol; 4 mM ATP; 200 μM each CTP, UTP, and GTP; 100 μM each dATP, dGTP, and dTTP; 20 μM [α-32P]dATP (10,000 cpm/pmol); and 0.23 μg of pSV01ΔEP DNA (ori1). Mixtures were incubated for 60 min at 37°C. One unit of elongation inhibitor activity was defined as the amount that inhibited the incorporation of dNMP by 50%. When products were analyzed by gel electrophoresis, reactions were terminated by the addition of 20 mM EDTA, 1% sodium dodecyl sulfate, and 40 μg of *Escherichia coli* tRNA as carrier, and the mixture was digested with proteinase K (100 μg/ml) at 37°C for 30 min. After extraction with phenol/chloroform (1:1, vol/vol), products were precipitated with ethanol, dried, and electrophoresed for 12 hr at 50 V in a 1.2% agarose gel containing 30 mM NaOH and 1 mM EDTA. The gel was then dried and autoradiographed.

The starting material for the isolation of the inhibitor was the 0.6 M PC fraction (fraction II), described by Prelich et al. (11), prepared from HeLa extract. This fraction (476 mg) was rechromatographed on a PC column (5 mg of protein per ml of bed volume: 2.5 × 20 cm) equilibrated with buffer A [25 mM Tris-HCl, pH 7.5/10% (vol/vol) glycerol/1 mM dithiothreitol/1 mM EDTA 0.01% Nonidet P-40/0.1 mM phenylmethylsulfonyl fluoride] containing 25 mM NaCl and was eluted stepwise with buffer A (180 ml per step) containing 0.3, 0.4, 0.6, and 0.8 M NaCl. All fractions were dialyzed against buffer A containing 20% (wt/vol) sucrose and 25 mM NaCl. The 0.6 M PC fraction, containing the inhibitor (119 mg; 33,000 units), was adjusted to 0.2 M NaCl, applied to a double-stranded-DNA (dsDNA)-cellulose column (8.0 mg of protein per ml of bed volume; 2.5 × 3.0 cm) equilibrated with buffer A containing 25 mM NaCl, and eluted stepwise with the same buffer (40 ml per step) containing 0.2, 0.4, 0.6, and 0.8 M NaCl. The inhibitory activity was eluted at 0.6 M NaCl and was dialyzed against buffer A containing 25 mM NaCl. This fraction (1.17 mg of protein, 4912 units) was concentrated by adsorption to a 0.8-ml dsDNA-cellulose column and eluted with 1.0 M NaCl in buffer A. This concentrated dsDNA-cellulose fraction (0.91 mg of protein, 2800 units) was dialyzed against buffer A containing 25 mM NaCl. An aliquot (0.2 ml, 0.13 mg of protein, 400 units) of the concentrated dsDNA-cellulose fraction was adjusted to 0.25 M NaCl and layered onto a 5.0-mllinear gradient of glycerol (15–35%, vol/vol) in buffer A containing 0.25 M NaCl. Gradients were centrifuged at 45,000 × g for 24 hr in a Sorval AH650 rotor at 4°C. Thirty fractions (0.17 ml) were collected from the bottom, and bovine serum albumin (Miles) was added to each fraction (0.2 mg/ml) immediately after collection in order to stabilize the activity. The inhibitor peaked at fraction 18 (from the bottom), which corresponded to a sedimentation coefficient of 4.2 S. The pool of fractions 16–20 yielded 60% of the activity applied to the gradient. The overall purification of the inhibitor was at least 100-fold with a yield of 5% starting from the 0.6 M PC fraction.

RESULTS

PCNA Is Not Required for Replication of SV40 DNA with Purified Proteins. SV40 T antigen, HeLa SSB, HeLa DNA polymerase α-prime complex, and topoisomerase I supported SV40 DNA replication in vitro (6, 9), and this reaction was unaffected by the addition of PCNA (Fig. 1A). Further, none of these purified preparations (=1 μg of protein) contained detectable PCNA as determined by immunoblotting techniques that could have detected as little as 20 ng of PCNA. However, the possibility that PCNA levels below this amount could have supported the replication reaction was examined. A human lupus serum monospecific for PCNA was previously shown to neutralize PCNA activity (24). This anti-PCNA serum had no effect on SV40 DNA replication with purified proteins (Fig. 1B). When topoisomerase II, RNase H, the 5' → 3' exonuclease, and DNA ligase were included in the above reaction mixture, both leading-strand synthesis and the production of covalently closed monomer circles occurred (9). The amount and the nature of the DNA products formed by this replication system were unaffected by the addition of PCNA or anti-PCNA serum (data not shown). These experiments indicated that the purified replication system was not contaminated with PCNA and did not require PCNA.

PCNA Is Required for Efficient Replication of SV40 DNA in Crude Extracts. In contrast to SV40 DNA replication with purified proteins, replication with crude fractions has been shown to be stimulated by PCNA (10, 25). Consistent with this observation, anti-PCNA serum inhibited SV40 DNA replication catalyzed by crude extracts of HeLa cells (Fig. 2A). Purified PCNA restored ~70% of the inhibited DNA synthesis, thus demonstrating that the inhibition by the anti-PCNA serum was caused specifically by the neutralization of PCNA in the crude extract (Fig. 2B). PCNA did not stimulate DNA replication in the absence of anti-PCNA serum, indicating that the crude extract contained saturating levels of PCNA.

Fractionation of Crude Extract Yields an Inhibitor Required for PCNA Activation. The fractionation of crude extracts of HeLa cells by PC chromatography yielded a 1 M NaCl PC fraction (see Materials and Methods), which, in the presence of SV40 T antigen and HeLa SSB, catalyzed the synthesis of short DNA fragments in the absence of PCNA and much longer products in the presence of PCNA (Fig. 3, lanes 1 and 2). When this fraction was rechromatographed on PC and step-eluted with increasing concentrations of NaCl, it was evident that an inhibitor of the replication reaction was resolved from the fractions containing DNA polymerase α. The fractions that were eluted at 0.3 M and 0.4 M NaCl contained 90% and 10%, respectively, of the DNA polymer-
of replication products were observed. The addition of the 0.4 M PC fraction and PCNA (Fig. 3, lanes 6 and 12) appeared to stimulate the amount of long product formed.

Most strikingly, the synthesis of long DNA products was reduced by the higher salt fractions, and it was the synthesis of the long products that was restored by PCNA. Thus, the inhibitor appears to block the elongation step of DNA synthesis, leading to the accumulation of short DNA fragments. These results are in keeping with the observations described in Figs. 1 and 2. The replication of SV40 DNA with purified proteins devoid of the inhibitor was unaffected by PCNA or antibodies against this protein, whereas cruder fractions, containing the inhibitor, required PCNA and were inhibited by neutralizing antibodies against PCNA.

The ability of the inhibitor to block the synthesis of SV40 DNA replication was used as an assay to further purify this activity, as described in Materials and Methods. The inhibitory activity (of either the dsDNA-cellulose or glycerol gradient fractions) was destroyed by heat and proteinase K treatment. Fractions containing the inhibitor activity also blocked a number of added to the reaction to occur at DNA termini, including the action of exonuclease III, the 5′ → 3′ exonuclease (9), and DNA ligase. The inhibition of these activities and the elongation inhibitor copurified, were heat-inactivated at the same rate, and were resistant to N-ethylmaleimide. Although the inhibitory activity was N-ethylmaleimide-resistant in all of these assays, the inhibition of DNA chain elongation by N-ethylmaleimide-treated inhibitor (dsDNA-cellulose) was not reversed upon addition of PCNA (data not shown).

**Effect of the Inhibitor on the Rate of Replication and the Size of DNA Products.** Reactions containing SV40 T antigen, HeLa SSB, topoisomerase I, and the 0.3 M PC fraction supported replication of SV40 DNA and accumulated products of increasing lengths at the same rate in the presence or absence of PCNA (Fig. 4). In the presence of the inhibitor, the rate and extent of DNA synthesis were markedly reduced, as was the size of the DNA products. In contrast, when reactions contained PCNA and the inhibitor, the rate of synthesis and the size of DNA products were almost the same as those observed in the absence of the inhibitor, though some differences in the size of products were noted at early times (5 and 10 min). These results were qualitatively similar.
to those described by Prellich and Stillman (10) with the exception that the accumulation of small products observed in Fig. 4 was governed by the inhibitor, and not by the absence of PCNA. PCNA affected chain elongation only in the presence of the inhibitor.

PCNA-Mediated Reversal of Inhibition Requires Additional Factors. When immunopurified DNA polymerase α-prime complex was used in place of the 0.3 M PC fraction, addition of the inhibitor decreased the size of the replication products, but this effect was not reversed by the addition of PCNA (Fig. 5, lanes 4–6). This indicated that one or more additional factors present in the 0.3 M PC fraction, but missing from the immunopurified polymerase α-prime complex preparation, were required for the inhibition to be reversed. This conclusion was verified by further fractionation of the 0.3 M PC fraction on DEAE-Sepharose, which separated the polymerase α-prime complex (eluted at 0.28 M NaCl, the 0.28 M DEAE fraction) from an activator fraction (eluted at 0.08 M NaCl). The activator fraction was further chromatographed on hydroxylapatite and eluted at a concentration of 0.5 M potassium phosphate. This fraction (0.5 M HAP), when combined with the inhibitor fraction and immunopurified polymerase α-prime complex, yielded small DNA products that were elongated in the presence of PCNA (Fig. 5, lanes 7 and 8). Similar results were obtained with the 0.28 M DEAE DNA polymerase α-prime complex that was isolated by further fractionation of the 0.3 M PC fraction (Fig. 5, lanes 13–17). In these experiments the effect of the inhibitor was not quantitatively reversed by the addition of the 0.5 M HAP fraction, due to a low recovery of the activator activity. In all reactions carried out with the inhibitor and activating fractions, the addition of aphidicolin completely prevented DNA synthesis, as did the omission of SV40 T antigen.

Further purification of the inhibitor by glycerol gradient centrifugation (see Materials and Methods) also resolved it from an activating factor that was required for the PCNA-mediated elongation reaction. The activation factor isolated from the 0.3 M PC fraction and the activator isolated from the inhibitor fraction appear to be different. Thus, the elongation phase of DNA synthesis that is carried out by the four purified proteins (SV40 T antigen, HeLa SSB, topoisomerase I, and polymerase α-prime) is blocked in the presence of an inhibitor we have isolated, and this inhibition is overcome by
the addition of PCNA and at least two other activating factors.

DISCUSSION
The results presented here support the conclusion that PCNA is required for SV40 DNA replication in vitro when the elongation of DNA chains is blocked by a protein inhibitor. This inhibitor also interferes with other enzymatic reactions occurring at the ends of DNA, such as those catalyzed by DNA ligase, exonuclease III, and the 5' → 3' exonuclease. This is consistent with the results of Prelich and Stillman (10), who noted that the absence of PCNA during SV40 DNA replication resulted in the inhibition of DNA ligase activity as well as DNA chain elongation. In contrast, the unwinding of SV40 ori+ DNA by the combined action of T antigen, SSB, and topoisomerase I (27, 28) was unaffected by the inhibitor (data not shown). This unwinding reaction depends solely on circular duplex DNA with no ends, whereas the enzymatic reactions affected by the inhibitor require free DNA termini. This suggests that the site of action of the inhibitor is at the ends of DNA chains.

The effects of the inhibitor on SV40 DNA replication carried out by four purified proteins can be overcome by PCNA and at least two other activating factors. Further, the block of exonuclease III activity by the inhibitor can be reversed by PCNA and the activating factors alone, in the absence of DNA polymerase activity (data not shown). We suggest that the inhibitor acts by blocking the ends of DNA chains, possibly by binding to them noncovalently, and that the inhibitor can be displaced from these ends by the combined action of PCNA and the activating factors. The inhibitor decreased the level of nucleotide incorporation as much as 75–80%. However, the PCNA-mediated synthesis of longer DNA products formed in the presence of the inhibitor did not necessarily lead to a level of nucleotide incorporation observed in the absence of the inhibitor. It is possible that this discrepancy may be resolved with further purification of the inhibitor and the activating factors.

At present, the requirements for the elongation of primed templates in eukaryotic systems are unclear. DNA polymerase α was assumed to be the only polymerase involved in replication. Recently, DNA polymerase δ has been proposed as an entity distinct from DNA polymerase α that is also involved in replication. These two polymerases were reported to differ immunologically and in some of their intrinsic properties (29–31). Further, some preparations of DNA polymerases δ have been shown to require PCNA for elongation activity (29). Nevertheless, some of the characteristics used to distinguish polymerase α from polymerase δ have been questioned, making the distinction between these enzymes less clear. For example, the presence of a 3' → 5' exonuclease and the resistance of chain elongation to the inhibitor N2-[p-(n-butyl)phenyl]-dGTP, properties thought to be specific for DNA polymerase δ, have also been observed with the 180-kDa catalytic subunit of DNA polymerase α isolated from Drosophila (32, 33). In contrast, the intact DNA polymerase α–primase complex of Drosophila did not contain 3' → 5' exonuclease activity and it was inhibited by low concentrations of N2-[p-(n-butyl)phenyl]-dGTP (28, 29). Further studies will be required to elucidate the differences between these two DNA polymerases and their roles in replication.

A number of studies have suggested an important role for PCNA in replication and in the control of cellular proliferation (17, 20, 21, 34). It is possible that PCNA, the inhibitor protein, and other activating factors essential for the elongation reaction constitute a set of proteins that may regulate DNA synthesis. It should be noted, however, that SV40 DNA replication is not under many of the controls operating in cellular DNA replication. It is possible that PCNA may play a more direct and complex role in replication than suggested by the results presented here. The further purification and characterization of the inhibitor and the other polymerases that lead to the PCNA-dependent elongation of DNA chains may offer a more tractable biochemical approach to the regulation of DNA synthesis than has been hitherto available.

We thank Ms. C. Turck and Ms. N. Belgado for their assistance. We thank Dr. Victoria Richon for help with the PCNA immunoblotting experiments. This work was supported by Grant GM34559 from the National Institutes of Health, P.B. and F.D.B. are supported by fellowships from the National Institutes of Health and the American Cancer Society, respectively.