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**Identification of the replicative intermediates in SV40 DNA replication *in vitro***

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Received 18 May 1984; Revised 2 July 1984; Accepted 13 July 1984

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**ABSTRACT**

The soluble replication system in which the exogenously added simian virus 40 (SV40) DNA can be replicated semiconservatively *in vitro*, has been developed (Ariga and Sugano, *J. Virol.* 48, 481, 1983). This paper further characterized the *in vitro* products synthesized on the cloned DNA containing the origin of SV40 DNA replication. The time course and pulse-chase experiments showed that the *in vitro* products were converted from the open circle to closed circles having the various superhelical densities, and finally to the twisted form I DNA seen *in vivo* by the analysis of agarose gel electrophoresis, alkaline sucrose gradient centrifugation, and density-transfer in isopycnic centrifugation. The replicative intermediates isolated after the short term incubation had replicated strands of the size smaller than the full length, most of which correspond to that of the putative Okazaki fragment. These and the previous results indicate that this *in vitro* system should be useful to investigate the molecular mechanism of SV40 DNA replication.

**INTRODUCTION**

The genome of simian virus 40 (SV40) represents a simple model for a single mammalian replicon and is a circular duplex molecule of about 5.2 kilobases. The complete nucleotide sequence of SV40 DNA is known (1, 2), and the structure and the genetic organization have been studied extensively (3, -6).

SV40 DNA replication begins at a unique site and proceeds bidirectionally (7, 8). The genetic analysis of the viral replication functions and the studies with purified T antigen which is the early gene product of SV40 enabled us to examine the genetic requirement and the minimum requirement of the origin sequence of the DNA replication; the origin of SV40 DNA replication has been mapped to a 60-65 base-pair region centered at the unique BglI site (7, 9), and binding of T antigen to the origin regulates the initiation of each of round of DNA replication (10, -13). Also,

the mode of replication has been studied by electron microscopic analysis of replicative intermediates (8) and by the analysis of distribution of radioactivity in pulse-labeled viral DNA (7).

Recently, we have developed an in vitro SV40 DNA replication system in which the initiation and bidirectional replication occur on the exogenously added SV40 DNA or cloned DNA containing SV40 origin of DNA replication. This system is consisted of a mixture of a soluble extract of HeLa cell nuclei and the cytoplasm from SV40-infected CosI cells (14, 15). The replication manner taken place in this system is quite similar to that of in vivo; the absolute dependence of SV40 T antigen and T antigen binding site II present in SV40 DNA and the involvement of DNA polymerase  $\alpha$  (14).

In the present paper, the replicative intermediates synthesized after a short term incubation were analyzed, showing that the several situations taken place in vitro were mimic to those in vivo.

### MATERIALS AND METHODS

#### Cells and virus

The SV40-transformed monkey cell CosI (15) was obtained from Y. Gluzman. SV40 was propagated in the monkey cell line GC7, and SV40 DNA was extracted from purified virions by CsCl-ethidium bromide equilibrium centrifugation as described previously (14).

#### Preparation of nuclear extract and cytoplasm

All of the following procedures have been described previously in detail (14). HeLa nuclei in hypotonic buffer were prepared by Dounce homogenization of cells which were grown in suspension culture. The nuclei, which were rapidly frozen in liquid nitrogen, were thawed and extracted with 100 mM NaCl at 0°C for 5 min. The HeLa nuclear extract was freed of insoluble material by centrifugation at 20,000 x g for 20 min. Cytoplasm was prepared from the monolayer cultures of CosI cells which had been infected for 40 h with SV40 at a multiplicity of infection of 100PFU per cell. Crude CosI cell cytoplasm was prepared by Dounce homogenization as previously described for adenovirus-infected HeLa cells (20). After centrifugation of crude cytoplasm at 100,000 x g for 30 min, the supernatant was precipitated with

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60%  $(\text{NH}_4)_2\text{SO}_4$ . The  $(\text{NH}_4)_2\text{SO}_4$  precipitate was dissolved in a solution of 25mM Tris (pH 7.5), 7mM  $\beta$ -mercaptoethanol, 0.1mM EDTA 10% glycerol, and 50mM NaCl, dialyzed against the same buffer, and used as cytoplasm in the following experiments.

#### Conditions for *in vitro* reaction

The DNA template used in this study was form I of pSVO which harbors the origin region of SV40 (0.64-0.71 map unit) in pBR322 (14). Reaction mixtures (100  $\mu$ l) contained 25mM HEPES (N-2 hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) (pH 7.5), 5mM  $\text{MgCl}_2$ , 0.5mM dithiothreitol, 0.05mM each dATP, dGTP, and dTTP, 1.5 $\mu$ M [ $\alpha$ - $^{32}\text{P}$ ] dCTP (410Ci/mmol, 5,000 to 15,000 cpm/pmole) 3.75mM ATP, 20 $\mu$ l of HeLa nuclear extract (protein concentration, 2.5mg/ml), 5 $\mu$ l of Cos-1 cytoplasm (protein concentration, 10mg/ml), and 0.4 $\mu$ g of pSVO DNA. After incubation of the reaction mixture for 60 min at 37°C and subsequent digestion with pronase (10 $\mu$ g) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol with or without phenol extraction. The DNA was dissolved in 50  $\mu$ l of 40mM Tris-1mM EDTA-5mM sodium acetate (pH 7.8) and electrophoresed on 1% agarose gels in the same buffer. The gels were dried and autoradiographed on Kodak X-ray film XRS.

## RESULTS

### Time course analysis of *in vitro* products

Time course experiment was carried out (Fig. 1). The radioactivity incorporated in an acid insoluble fraction was linear up to 20 min of incubation and gradually decreased until 90 min. The *in vitro* products synthesized in the same experiments were visualized on a neutral agarose gel after the autoradiography (Fig. 2). After first 5 min of the reaction, main product synthesized was form II which was the open circle DNA. After 10 min of the reaction, the closed circular DNAs having different superhelical densities appeared in addition to an increase in the form II DNA. After 20 min of the reaction, the closed circular DNA which has the complete helical turns seen *in vivo*, in addition to the several oligomer (presumably dimer and trimer), could be seen. The phenomenon at 40 and 60 min incubation were similar to that at 20 min incubation. These results shows that the forms of the *in vitro* product were from the full size open circular to

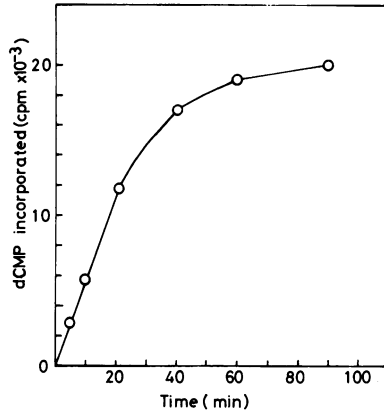


Fig. 1. Time course experiment of DNA synthesis in vitro on pSVO.

The reaction was carried out on 0.4  $\mu\text{g}$  of pSVO as a template. After 0, 5, 10, 20, 40, 60, 90 min at 37°C, the acid-insoluble radioactivity was counted.

closed circular having the various superhelical turns, and finally to the complete form I DNA observed in vivo, probably via several concatemers.

#### Pulse-chase experiments of in vitro reaction

Pulse-chase experiments were carried out (Fig. 3). After the incubation of the mixture for 5 min (pulse), two hundred fold of cold dCTP was added to the mixture, followed by the further incubation for 60 min (chase). The in vitro products were analyzed by neutral agarose gel electrophoresis (Fig. 3A), alkaline sucrose gradient centrifugation (Fig. 3B), and neutral isopycnic centrifugation (Fig. 3C). An analysis by a neutral agarose gel showed that a half of pulse labeled circular DNAs was converted to the form I after the 60 min chase (Fig. 3A). These phenomenon were much clearly shown by a sedimentation analysis in an alkaline sucrose gradient, where the DNAs smaller than the full length pSVO DNA, which had the size of the putative Okazaki fragment, were converted to the forms of full sized DNAs corresponding to the closed circular and open circular (Fig. 3B). The process of DNA replication was also observed in the density shift experiment, in which the DNA was synthesized in the reaction mixture containing dBrUTP in place of dTTP and analyzed by neutral

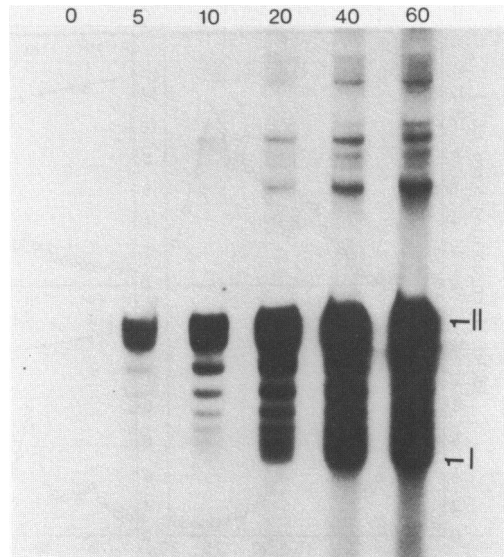


Fig. 2. Time course experiment of DNA synthesis *in vitro* analyzed by the neutral agarose gel electrophoresis.

The reaction was carried out on 0.4  $\mu\text{g}$  of pSV0 as a template. After incubation of the reaction mixture for 0, 5, 10, 20, 40, 60 min at 37°C and subsequent digestion with proteinase K (10  $\mu\text{g}$ ) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol. The DNA was dissolved in 50  $\mu\text{l}$  of 40 mM Tris-1mM EDTA-5mM sodium acetate (pH7.8) and electrophoresed on 1% agarose gels in the same buffer. The numbers shown on the top of the figure represent the incubation time of the mixture. The horizontal bars represent the positions of form II and I DNA from the top.

CsCl equilibrium centrifugation. Almost all the DNA banded at the position of the unsubstituted density were converted to that between the fully substituted and halfly substituted densities (Fig. 3C). This indicates that DNA synthesized during a short time incubation was elongated to the full sized molecules, and that some of the molecules completing the one round of replication got in reinitiation of the DNA replication.

#### Identification of replicative intermediates in *in vitro* reaction

It is already known that when infected cells are pulse-labeled for a short time and DNA from the Hirt supernatant (16) is analyzed, labeled viral DNA sediments in a broad peak slightly faster than form I DNA on neutral sucrose gradients (17, 18). Sedimentation

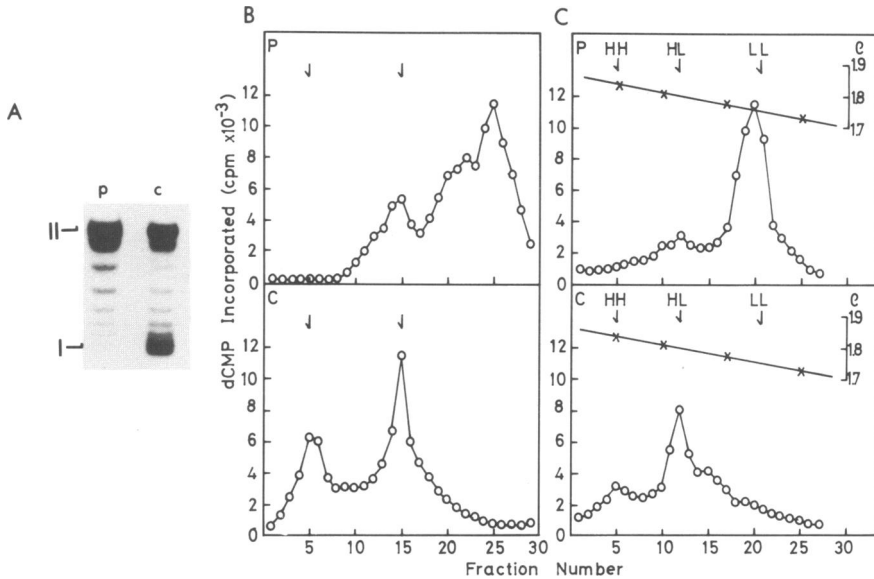


Fig. 3. Pulse-chase experiment of DNA synthesis *in vitro*.

The DNA synthesis was carried out on 0.4  $\mu\text{g}$  of pSVO as a template. After the reaction for 5 min (pulse), 200 fold concentration of cold dCTP (300 $\mu\text{M}$ ) were added to the mixture and the reaction was continued up to 60 min (chase). After the reaction, DNA was extracted with phenol and precipitated with ethanol.

P: pulse ; C: chase.

A: The samples were analyzed by the neutral agarose gel electrophoresis as in Fig. 2. The horizontal bars represent the positions of form II and I DNA from the top.

B: The samples were loaded onto 5 to 20% alkaline sucrose gradient containing 0.2M NaOH, 0.5M NaCl, and 10mM EDTA and centrifuged in an SW50.1 rotor at 45,000 rpm for 3 hr at 4°C. The acid-insoluble radioactivity was counted after fractionation. The arrows show the positions of the denatured supercoiled and open circular pSVO DNAs. In this condition denatured open circular and linear DNA formed a single peak.

C: The products were analyzed by the isopycnic centrifugation at a neutral pH. A standart reaction mixture containing 30 $\mu\text{M}$  each dATP, dGTP, and dBrUTP (in place of dTTP) and 1.5 $\mu\text{M}$  [ $^3\text{P}$ ] dCTP was carried out on pSVO. The DNA was precipitated with ethanol as in Fig. 3A and B. The density of the DNA sample containing 25mM Tris (pH7.4), 1mM EDTA, and 0.1M NaCl was adjusted to 1.8  $\text{g}/\text{cm}^3$  by the addition of CsCl and centrifuged in a SW50.1 rotor at 43,000 rpm for 48 hr at 25°C. After fractionation, the acid-isoluble radioactivity was counted, and the density was measured. The arrows indicate the expected positions for fully substituted (HH), hybrid (HL), and unsubstituted (LL) DNAs.

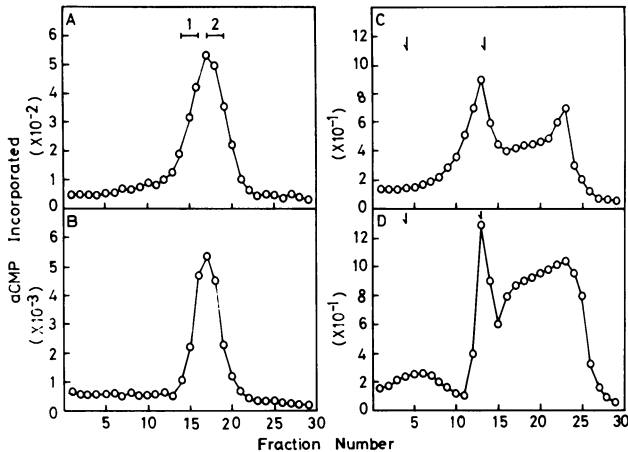


Fig. 4. Isolation of the replicative intermediates of the reaction. The DNA synthesis was carried out on 0.4  $\mu\text{g}$  of psVO as a template. After the reaction for 5 min (A) and 60 min (B), DNA was extracted with phenol and precipitated with ethanol. The DNA samples were loaded onto 5 to 20% neutral sucrose gradient containing 0.1M NaCl, 0.1M EDTA, and 10mM Tris (pH 7.4) and centrifuged in an SW50.1 rotor at 45,000 rpm for 3 hr at 4°C. The acid-insoluble radioactivity of 70  $\mu\text{l}$  of the aliquot was counted after fractionation. In case of the 5 min reaction, the fractions from 13 to 16 and those from 17 to 19 were pooled in pool 1 and 2, respectively, and DNA in the pooled fractions was precipitated with ethanol. The pooled DNA samples were further analyzed by the alkaline sucrose gradient centrifugation as in Fig. 3B. Fig. 4C and D show the sedimentation patterns of pool 1 and 2 DNA, respectively, on alkaline sucrose gradients. The arrows show the positions of the denatured supercoiled and open circular psVO DNAs.

of these replicative intermediates in alkaline sucrose gradients allows separation of the newly synthesized strands, which are shorter than or equal to the length of a complete viral DNA strands (19). To characterize the replicative intermediates synthesized *in vitro*, the intermediates were sedimented through the neutral sucrose gradients (Fig. 4). The distribution pattern of the DNA product synthesized in a 5 min reaction was a little broader than in a 60 min reaction (Fig. 4A and B). In order to separate DNAs between relatively early and late intermediates, the faster sedimenting (early intermediate) and the slower sedimenting DNA (late intermediate) in a 5 min reaction were collected in pool 1 and 2, respectively, and analyzed by

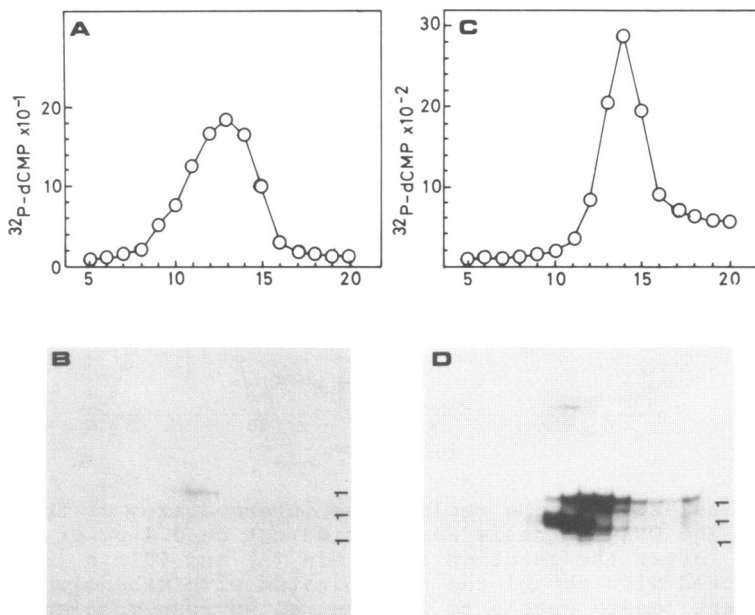


Fig. 5. Fractionation analysis of the replicative intermediates.

After the reaction on pSVO, DNA was extracted with phenol and precipitated with ethanol. The samples were loaded onto 5 to 20% neutral sucrose gradients as in Fig. 4. The acid-insoluble radioactivity of 25  $\mu$ l of aliquot was counted after fractionation. The DNA in the rest of sample was precipitated with ethanol and visualized by autoradiography after agarose gel electrophoresis. (A). the patterns of sucrose gradients in a reaction for 5 min. (B). the patterns of agarose gel in a reaction for 5 min. (C). sucrose gradients in a reaction for 60 min. (D). agarose gel in a reaction for 60 min. The horizontal bars in Fig. B and D represent the positions of form II, III, and I DNA from the top.

sedimentation through the alkaline sucrose gradients (Fig. 4C and D). The pooled DNAs gave the similar distribution patterns of DNAs which are shorter than or equal to the length of the complete DNA strands. However, the faster sedimenting DNAs on the neutral sucrose gradient had lesser amount of the intermediate sized DNAs located between the complete DNA and the putative Okazaki fragment than that of the slower sedimenting DNAs. These patterns clearly indicate the different size of newly synthesized strand between early intermediate and late intermediate. The results clearly show that the replicative intermediates synthesized in vitro are



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quite similar to those synthesized in vivo.

Fractionation analysis of the in vitro products followed by gel electrophoresis.

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In order to confirm the relationship between size and structure of the replicative intermediates, the fractionation studies were performed. After the reaction for 5 or 60 min, the in vitro product DNAs were extracted from the mixture and separated by the neutral sucrose gradients (Fig. 5A and C). The similar results as those in Fig. 4A and C were obtained. DNA in each fraction was precipitated with ethanol and analyzed by neutral agarose gel electrophoresis (Fig. 5B and D). The preparations used in Fig. 5 gave rise to lesser amounts of form I DNA observed in vivo as compared to those used in Fig. 1 to Fig. 4. These phenomenon sometimes occurred preparations by preparations. However, it is very clear that the peak fraction of incorporated radioactivity at 5 min reaction gave just form II DNA, while the peak fraction at 60 min reaction gave the various form I DNAs in addition to form II and III DNA (Fig. 5B and D). Furthermore, at 60 min reaction, the faster sedimenting fraction ahead of the main peak of the incorporated radioactivity gave rise to mainly form III DNA in addition to several concatemers (Fig. 5D), suggesting that form III DNA, concatemers may be precursors of the final daughter molecules. These situations of replicating DNAs are quite similar to those observed in vivo.

#### DISCUSSION

The present paper clearly identified the replicative intermediates synthesized in vitro on the exogenous template DNA containing SV40 origin of DNA replication. The structural features of these intermediate DNAs were quite similar to the in vivo intermediates in terms of the sedimentation profiles at both neutral and alkaline pH. The pulse-labeled DNAs can be chased into form I or form II DNAs. Therefore these are true precursors to the mature DNA. Several characteristics of this in vitro system described in this and previous communications (14) support its biological relevances including the absolute dependence on SV40 T antigen and T antigen binding site II, the initiation of DNA replication from the origin, the appearance of the typical

replicative intermediates, and the involvement of the DNA polymerase  $\alpha$  in the reaction. Therefore this in vitro system should be useful for the purification and subsequent characterization of viral and cellular proteins involved in SV40 DNA replication.

#### ACKNOWLEDGEMENT

This work was supported by grants from the Ministry of Education, Science, and Culture, Japan. I am grateful to Miss E. Ono for beautiful typing of this manuscript. And I am indebted to Dr. N. Yamaguchi for the critical reading of this manuscript.

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